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Über die Extraktivstoffe des Fischfleisches und der Muscheln¹.

VON

U. Suzuki und Mitarbeitern.

Die vorliegende Arbeit ist die Fortsetzung einer Mitteilung, die vor drei Jahren in HOPPE-SEYLER'S Zeitschrift für physiologische Chemie Bd. 62 Heft 1 (1909) erschienen ist.

Die Methoden, die wir zur Isolierung der stickstoffhaltigen Stoffe anwendeten, waren im grossen und ganzen dieselben, wie in der früheren Mitteilung. Deshalb haben wir die genaue Beschreibung derselben nicht angegeben.

Untersucht wurden das frische Fleisch von Sardinen, Maguro (*Thynnus thunnus*), Tai (*Pagrus major*), Krabben, Hamaguri (*Cytheria meretrix* L.) und Austern.

Als Hauptergebnisse dieser Arbeit haben wir aus Krabben- und Taifleisch eine Base "Kanirin" $C_6H_{14}N_2O_2$ isoliert, die mit dem Lysin nicht identisch ist. Ferner wurde die Verbreitung von Betain, Tryptophan, Imidazoläthylamin, Histidin, Karnosin, Alanin, Tyrosin, Leucin, Prolin, u. s. w. nachgewiesen.

I. Sardinen.

Von U. SUZUKI und M. MIHATA.

2 Kilo frisches, von Haut, Gräten und inneren Organen befreites Fleisch von Sardinen wurden dreimal mit warmem Wasser (50—60°) extrahiert. Die Auszüge betrugen im Ganzen 3,4 l.

¹ Diese Arbeit ist schon vor einigen Jahren in The Journal of the Tokyo Chemical Society publiziert worden. Vol. 30. No. 9. (1909) Vol. 31. No. 6. u. No. 7. (1910).

[Jour. Coll. Agric., Vol. V. No. 1, 1912.]

Die quantitative Bestimmung gab folgendes Resultat:

	In 100 Teilen Trockensubstanz des Extraktes.	Gesamtstickstoff als 100 berechnet.
Gesamtstickstoff	4,32	100,00
Eiweisstickstoff	1,32	30,55
Basenstickstoff	2,24	50,18
Stickstoff in anderer Form	0,76	19,27

Der wässrige Extrakt wurde mit Essigsäure schwach angesäuert (bis die Flüssigkeit ca. 2% Essigsäure enthielt) und mit Tannin versetzt. Das Filtrat vom Tannin-Niederschlag wurde durch Baryt von Tannin befreit, mit Schwefelsäure angesäuert und mit Phosphowolframsäure gefällt.

A. DER PHOSPHOWOLFRAMSÄURE-NIEDERSCHLAG.

Man zerlegte den Niederschlag in bekannter Weise mit Baryt und entfernte den Überschuss von Baryt durch Schwefelsäure. Die so erhaltene alkalische Flüssigkeit wurde mit Kohlensäure gesättigt und mit Quecksilberchlorid gefällt.

(1). Der Quecksilberchlorid-Niederschlag (Histidin). Aus diesem Niederschlag erhielt man das reine Histidin als salzsauren Methylester. Zu diesem Zwecke wurde der Niederschlag durch Schwefelwasserstoff zerlegt, vom Quecksilbersulfid abfiltriert und bei vermindertem Druck bis zum Trocknen eingedampft. Der zurückgebliebene Syrup wurde mit wenig Methylalkohol versetzt und wieder eingedampft, um das Wasser vollständig auszutreiben. Man setzte dann wasserfreien Methylalkohol zu und leitete trockenes Salzsäuregas bis zur Sättigung ein. Nach dem Eindampfen im Vakuum schied sich das Histidin als methylester-salzsaures Salz aus. Die Ausbeute betrug 5,8_g. Zur Reinigung wurde das Salz in heissem Methylalkohol gelöst und durch Zusatz von Aethylalkohol und Aether ausgeschieden.

0,1040 _g Subst. gaben	0,0303 _g Cl
0,1238 _g „ „	19,2 ^{c.c.} N (19° 758 ^{mm})
0,1424 _g „ „	0,1804 _g CO ₂ 0,0714 _g H ₂ O

	C	H	N	Cl
C ₇ H ₁₁ N ₃ O ₂ . 2H Cl. Ber.	34,67	5,45	17,36	29,34
Gef.	34,48	5,64	17,78	29,13

Es entspricht also dem salzsauren Methylester des Histidins. Das Salz besteht aus farblosen Prismen; zersetzt sich bei 198° (unkorr.) unter Schäumen. Es ist in Wasser und in Methylalkohol leicht, in Aethylalkohol etwas schwerer und in Aether nicht löslich. Die wässrige Lösung gibt eine weisse Fällung mit Millon'schem Reagenz und eine intensive rote Färbung mit alkalischer Diazobenzolsulfonsäure. Sie gibt auch Biuretreaktion beim Erwärmen.

0,589_g Methylestersalz in 9,9708_g Wasser gelöst, drehte im 10^m Rohr das Natriumlicht $0,73^{\circ}$ nach rechts. Das spez. Gew. der Lösung war 1,0163.

$$\text{Mithin } [\alpha]_D = + 14,5^{\circ}$$

Wird das methylester-salzsaure Salz des Histidins in wenig Wasser gelöst und langsam eingedampft, so wird der Ester zum Histidindichlorid verseift, welches aus heisser methylalkoholischer Lösung durch Zusatz von Aether als farblose Prismen sich ausscheidet. Es schmilzt bei 234° (unkorr.) ohne Schäumen. Für die Analyse wurde es im Vakuum bei 100° getrocknet.

0,1485_g Subst. gaben 0,0459_g Cl
0,1372_g „ „ 21,4^{c.c.} N (19° . 765^{mm})

		N	Cl
$C_6 H_9 N_3 O_2 \cdot 2 H Cl$	Ber.	18,42	31,11
	Gef.	18,05	30,95

0,6981_g Histidindichlorid in 19,869_g Wasser gelöst, bei einem sp. Gew. von 1,0144, drehte im 20^{cm} Rohr das Natriumlicht $1,04^{\circ}$ nach rechts.

$$\text{Mithin } [\alpha]_D = + 15,1^{\circ}$$

(2). Das Filtrat vom Quecksilberchlorid-Niederschlag des Histidins wurde mit Silbernitrat und Baryt gefällt. Der braune Niederschlag wurde mit Schwefelwasserstoff zerlegt, bei vermindertem Druck stark eingedampft und unmittelbar mit Pikrinsäure erwärmt. Nach dem Erkalten schied sich das Kreatininpikrat als hellgelbe Nadeln aus. Die Ausbeute betrug 1,9_g. Für die Analyse wurde es aus heissem Wasser umkristallisiert und im Vakuum bei 100° getrocknet.

0,1582_g Subst. gaben 32,7^{c.c.} N (15° . 761^{mm})
0,1324_g „ „ 0,1708_g CO₂ 0,0422_g H₂O

	<i>C</i>	<i>H</i>	<i>N</i>
$C_4 H_7 N_3 O \cdot C_6 H_3 N_3 O_7$. Ber.	35,08	2,92	23,54
Gef.	35,18	3,58	24,24

Das Pikrat zersetzt sich bei 214° (unkorr.) In kaltem Wasser ist es schwer löslich. Die wässrige Lösung gibt eine rote Färbung durch Zusatz von Alkalien.

Aus dem Pikrate wurde das Platinchloriddoppelsalz des Kreatinins dargestellt.

0,2031_g Subst. gaben 0,0637_g *Pt*

Pt

$(C_4 H_7 N_3 O \cdot HCl)_2 PtCl_4$ Ber. 31,03

Gef. 31,35

Ferner wurde das charakteristische Zinkchloriddoppelsalz des Kreatinins dargestellt.

(3). Das Filtrat vom Silbernitrat und Baryt-Niederschlag wurde in gewöhnlicher Weise wieder mit Phosphowolframsäure gefällt. Nach Zerlegung des Niederschlages erhielt man 3,7_g Karnosin-pikrat, welches aus heissem Wasser umkristallisiert, im Vakuum bei 100° getrocknet und analysiert wurde.

0,5083_g Subst. gaben 0,2582_g Pikrinsäure

0,1082_g „ „ 19,4^{c.c.} *N* (13°. 764^{mm})

0,1282_g „ „ 0,1287_g *C O*₂ 0,0455_g *H*₂ *O*

C *H* *N* Pikrinsäure

$C_9 H_{14} N_4 O_3 \cdot C_6 H_3 N_3 O_7$ Ber. 39,56 3,74 21,54 50,33

Gef. 39,75 3,98 21,31 50,81

Im Kapillarrohr erhitzt wird es gegen 200° braun und zersetzt sich bei 218° (unkorr.)

Das Nitrat des Karnosins besteht aus farblosen Prismen, schmilzt bei 211° (unkorr.) Es löst sich in Wasser leicht, etwas schwerer in Alkohol und ist fast unlöslich in Aether.

0,4136_g Subst. gaben 0,5357_g Nitronnitrat (nach Nitron-Verfahren)

*H N O*₃

$C_9 H_{14} N_4 O_3 \cdot H N O_3$ Ber. 21,80

Gef. 21,75

B. DAS FILTRAT VOM PHOSPHOWOLFRAMSÄURE-NIEDERSCHLAG.

Das Filtrat vom Phosphowolframsäure-Niederschlag wurde durch Baryt von Phosphowolframsäure und Schwefelsäure befreit und bei niederem Druck stark eingedampft. Man erhielt dabei 1,7_g Tyrosin.

0,1524_g Subst. gaben 0,3352_g C O₂ 0,0867_g H₂ O
 0,1785_g „ „ 11,8^{c.c.} N (18°, 766^{mm})

		C	H	N
C ₉ H ₁₁ N O ₂	Ber.	59,67	6,07	7,73
	Gef.	59,98	6,38	7,67

Es bildet seidenglänzende Nadeln, ist ziemlich schwer löslich in Wasser, gibt sowohl starke Millon'sche Reaktion, wie auch Pauly'sche Diazoreaktion.

0,5763_g Tyrosin in 23,630_g 10%iger Salzsäure gelöst, bei einem sp. Gew. von 1,016, drehte das Natriumlicht 0,63° nach links.

$$\text{Mithin } [\alpha]_D = -14,8^\circ$$

Es war also l-Tyrosin.

Die Mutterlauge des Tyrosins lieferte, nach der Estermethode verarbeitet, 0,23_g Leucin. Aus heissem Wasser umkristallisiert, bildete es glänzende schuppenförmige Kristalle von schwach bitterem Geschmack. Zur Analyse reichte das Material nicht aus.

Aus 2 Kilo frischem Sardinenfleisch wurden isoliert:

Histidinmethylester	5,8 _g
Kreatininpikrat	1,9 _g
Karnosinpikrat	3,7 _g
Tyrosin	1,7 _g
Leucin	0,23 _g

II. Maguro (Thynnus thynnus).

VON U. SUZUKI und S. ŌTSUKI.

Schon vor zwei Jahren hat einer von uns gemeinsam mit M. YAMAKAWA aus frischem Magurofleisch, Kreatin, Histidin und Karnosin isoliert und das Vorhandensein von Alanin nachgewiesen. Bei der Wiederholung dieses

Versuches mit etwas modifizierter Methode ist es uns gelungen, ausser den oben genannten Stoffen noch Kreatinin und Imidazoläthylamin zu isolieren. Zu diesem Zwecke wurden 3 Kilo frisches Magurofleisch (=900_g Trockensubstanz) wiederholt mit warmem Wasser extrahiert. Der gesamte Extrakt, der ungefähr 12 Liter betrug, wurde mit einer 20%igen Tanninlösung versetzt. Der dabei entstandene dickbraune Niederschlag, der den Hauptanteil des Imidazoläthylamins mitreisst, wurde abgesaugt und mit 5%iger Schwefelsäure verrieben. Ein Teil des Niederschlages wurde dabei gelöst und die letztgenannte Base ging in Lösung über. Man filtrierte nun ab, und versetzte mit überschüssigem Baryt, um Tannin und Schwefelsäure zu entfernen. Die vom entstandenen Niederschlag abfiltrierte Flüssigkeit wurde nach dem Entfernen des Baryts mittels Schwefelsäure bei vermindertem Druck bis auf 100° eingedampft, mit wenig Tierkohle entfärbt und mit Pikrinsäure versetzt. Es schied sich dabei das Pikrat des Imidazoläthylamins aus, welches aus heissem Wasser umkristallisiert, gelbbraune, glänzende, rhombische Tafeln bildete. Die Ausbeute betrug *ca.* 2,5_g. Es löste sich in kaltem Wasser schwer. Im Kapillarrohr erhitzt, verwandelte sich die Farbe gegen 210° allmählich zu schwarzbraun und zersetzte sich bei 220° (unkorr.) unter Schäumen.

Für die Analyse wurde das Pikrat im Vakuum bei 100° getrocknet.

0,1522_g Subst. gaben 0,2017_g C O₂ 0,0390_g H₂ O

0,1349_g „ „ 25,6^{c.c.} N (15° 756^{mm})

0,4525_g „ „ 0,3660_g Pikrinsäure

$C_5 H_9 N_3 (C_6 H_3 O_7)_2$		C	H	N	Pikrin- säure
	Ber.	35,86	2,63	22,14	80,50
	Gef.	36,14	2,85	22,11	80,88

Aus dem Pikrate wurde das Platinchlorid doppelsalz der Base dargestellt. Es bestand aus gelbbraunen Prismen, die keinen Schmelzpunkt hatten. Im Kapillarrohr erhitzt, verkohlte es gegen 240° ohne Schäumen.

Analyse des Platinchloriddoppelsalzes (im Vakuum bei 100° getrocknet)

0,2070_g Subst. gaben 0,0775_g Pt

0,1644_g „ „ 12,2^{c.c.} N (18,5° 755^{mm})

		<i>N</i>	<i>Pt</i>
$C_5 H_9 N_3, H_2 Pt Cl_6$	Ber.	8,07	37,47
	Gef.	8,49	37,44

Das Nitrat der Base bestand aus farblosen Prismen, welche in Wasser leicht, in Alkohol etwas schwerer und in Aether unlöslich waren. Es schmolz bei 145° zu einem durchsichtigen Öle und zersetzte sich bei 165° (unkorr.) unter Schäumen. Die Bestimmung der Salpetersäure in dem Nitrat wurde nach dem Nitron-Verfahren ausgeführt.

Analyse des Nitrats (im Vakuum bei 100° getrocknet):—

0,1573_g Subst. gaben 0,500_g Nitron-nitrat

		$H N O_3$
$C_5 H_9 N_3 \cdot 2 H N O_3$	Ber.	53,2
	Gef.	53,0

Die Analyse stimmt also mit der Formel $C_5 H_9 N_3$, dem Imidazoläthylamin überein. Da das Magurofleisch reich an Histidin ist, so ist es wohl möglich, dass es durch CO_2 -Abspaltung in die letztgenannte Base übergeht. Bloss die Frage, ob diese Umwandlung im lebendigen Gewebe, ohne Bakterienwirkung stattfindet, können wir noch nicht entscheiden. Dass es durch Fäulnisprozess aus Histidin entsteht, ist neulich von D. ACKERMANN¹ festgestellt worden. Auch K. YOSHIMURA² hat diese Base aus faulenden Sojabohnen nachgewiesen.

Das Filtrat vom Tannin-Niederschlag wurde mit überschüssigem Baryt versetzt, vom entstandenen Niederschlag abfiltriert und nach dem Entfernen des Baryts mittels Schwefelsäure, bei vermindertem Druck, stark eingedampft und mit Alkohol versetzt. Nach einigen Tagen schieden sich 9,56_g reines Kreatin aus.

Die Mutterlauge des Kreatins wurde mit gesättigter Quecksilberchlorid lösung und Natriumacetat versetzt. Der entstandene weisse Niederschlag wurde abgesaugt, mit verdünnter Salzsäure erwärmt und abfiltriert. Das Filtrat wurde durch Einleiten des Schwefelwasserstoffs vom Quecksilber

¹ D. ACKERMANN: HOPPE-SEYLER's Zeitschrift f. Physiol. Chem. Bd. 65. Heft 6. (1910). Vergl. auch ACKERMANN und H. KUTSCHER: Z. f. Biologie Bd. 54 pag. 387.

² K. YOSHIMURA: Biochem. Zeitsch. 28. 16 (1910).

befreit, und bei verminderten Druck eingedampft. Es schieden sich dabei farblose Kristalle aus, welche zum Teil aus anorganischen Salzen bestanden. Durch Behandlung mit warmem Methylalkohol gelang es die organischen Substanzen von den anorganischen zu trennen. Nach dem Eindampfen der methylalkoholischen Lösung schied sich zuerst das salzsaure Kreatinin aus. Die Mutterlauge desselben lieferte bei weiterem Einengen und Verreiben mit absolutem Alkohol 10,44_g salzsaures Histidin. Von der Mutterlauge des Histidinchlorids erhielt man noch etwas Kreatinin und *ca* 1,5_g Imidazoläthylamin als pikrinsaure Salze. Durch fraktionierte Kristallisation konnte man die beiden Salze von einander trennen.

Das Kreatininpikrat bestand aus gelben Nadeln, mit dem Schmelzpunkt 211—212° C (unkorr.) Die Analyse desselben gab folgendes Resultat:—

0,1049 _g Subst. gaben	21,8 ^{c.c.} N (10° 758 ^{mm})		
0,1561 _g „ 0,1969 _g C O ₂	0,0510 _g H ₂ O		
	C	H	N
C ₄ H ₇ N ₃ O, C ₆ H ₃ N ₃ O ₇ Ber.	35,08	2,92	24,54
Gef.	35,01	3,69	24,71

Das Imidazoläthylaminpikrat:

0,1550 _g Subst. gaben	0,2043 _g C O ₂	0,0411 _g
0,1121 _g „	21,7 ^{c.c.} N (18° 752 ^{mm})	
0,5653 _g „	0,4590 _g Pikrinsäure	

C ₅ H ₉ N ₃ , 2 C ₆ H ₃ N ₃ O ₇	Ber.	C	H	N	Pikrin- säure
	Gef.	35,86	2,63	22,14	80,50
		35,95	2,96	22,12	81,04

Das Platinchloriddoppelsalz.

0,1978_g Subst. gaben 9,0742_g Pt.

$C_5 H_9 N_3, H_2 Pt Cl_6$ Ber.	Pt
	37,47
Gef.	37,51

Man sieht also, dass das Imidazoläthylamin teils durch Tannin und teils durch Quecksilberchlorid und Natriumacetat gefällt wird.

Aus 3 kg frischem Fleisch wurden isoliert:

Imidazoläthylaminpikrat	4,0 _g
Kreatin	9,56
Kreatininpikrat	Vorhanden
Histidin-Salzsaures Salz	10,44

III. Krabben.

VON U. SUZUKI, R. INOUE UND K. C. BHARATKAR.

Über Krabbenextrakt liegt schon eine ausführliche Mitteilung von D. ACKERMANN und F. KUTSCHER¹ vor. Die beiden Autoren haben nämlich eine ganze Reihe Stickstoffverbindungen aus frischem Krabbenfleisch dargestellt: Tyrosin, Leucin, Arginin, Lysin, Hypoxanthin, Betaïn, Pyrimidinmethylchlorid, Crangitin ($C_{13}H_{20}N_2O_4$) Crangonin ($C_{13}H_{26}N_2O_3$) und Neosin ($C_6H_{16}NO.Cl, Au Cl_3$). Die von uns untersuchte sogenannte "Grosse Krabbe" aus *Echizen* gab aber etwas andere Ergebnisse. Wir haben ausser Tyrosin, Alanin, Leucin und Arginin eine neue Base gefunden, die der Formel $C_6H_{14}N_2O_2$ entspricht. Da diese Base kein Methylester bildet, so kann sie nicht mit dem Lysin identisch sein. Wir haben für sie den Namen "Kanirin" vorgeschlagen. Ferner haben wir das Vorhandensein von Tryptophan nachgewiesen.

4 Grosse Krabben, die in frischem Zustande 3700_g wogen, lieferten 2087_g Fleisch, welches gleich mit warmem Wasser (60—70°) extrahiert wurde. Der wässrige Extrakt betrug im ganzen 6350^{c.c.}. Mit einem Teile des Extraktes wurde zuerst die quantitative Bestimmung ausgeführt.

	Im ganzen Extrakt (6350 ^{c.c.})	In 100 Teilen Trockensubstanz des Extraktes.
Trockensubstanz	163,32 _g	100,00
Gesamtstickstoff	25,81	15,81
Eiweiss „	3,62	2,22
Basen „	8,11	4,97
Ammoniak „	0,38	0,23
Stickstoff in anderer Form	13,70	8,40

¹ Zeits. f. Unters. v. Nahrungs-u. Genussmitteln. 1907. 13. 180, 610, 913 und 14. 687.

Der andere Teil diente zur Isolierung der einzelnen Stickstoffverbindungen. Zu diesem Zwecke wurde der Extrakt mit Tannin gefällt, vom entstandenen Niederschlag abfiltriert und nach dem Entfernen des Tannins durch Baryt und den Überschuss des Baryts mittels Schwefelsäure, bei niederem Druck stark verdampft. Da das Vorhandensein von Tyrosin und Tryptophan in dieser Flüssigkeit nachgewiesen war, so wurde es versucht nach Hopkin'schem Verfahren Tryptophan zu isolieren, indem die Flüssigkeit mit Schwefelsäure angesäuert, bis sie 5% der Säure enthielt, und mit Hopkin'schem Reagenz (Quecksilbersulfat in 5%iger Schwefelsäure) gefällt wurde. Der weisse, flockige Niederschlag wurde abgesaugt, mit 5%iger Schwefelsäure gewaschen, in Wasser verteilt und mit Schwefelwasserstoff zerlegt. Die vom Quecksilbersulfid abfiltrierte Flüssigkeit hinterliess nach dem Verdampfen im Vakuum einen braunen Sirup, welcher nach einigen Tagen etwas kristallinisches Tryptophan lieferte. Die Hauptmenge des Sirups blieb jedoch amorph, sodass die Menge des gereinigten Tryptophans für die Analyse nicht ausreichte. Die mit Essigsäure schwach angesäuerte Lösung des Tryptophans gab durch Zusatz von einigen Tropfen Bromwasser eine charakteristische rotviolette Färbung. Beim Schütteln mit Amylalkohol ging der Farbstoff in das letztere Reagenz über. Das Filtrat vom Quecksilbersulfat-Niederschlag des Tryptophans wurde durch Schwefelwasserstoff vom Quecksilber befreit und nach dem Austreiben des Schwefelwasserstoffes mit Schwefelsäure angesäuert und mit Phosphowolframsäure gefällt. Der Niederschlag wurde in gewöhnlicher Weise durch Baryt zerlegt. Die in der Weise dargestellte alkalische Flüssigkeit enthielt nur wenig Histidin und Kreatinin und Spuren von Purinbasen, darum wurde die Flüssigkeit unmittelbar mit Silbernitrat und Baryt gefällt. Aus diesem Niederschlag wurden 2_g Argininpikrat erhalten.

0,1066_g Subst. gaben 22,2^{c.c.} N (26° 760^{mm})

		N
$C_6 H_{14} N_4 O_2 \cdot C_6 H_3 N_3 O_7$	Ber.	24,32
	Gef.	24,28

Da es in jeder Beziehung mit reinem Argininpikrat vollständig identisch war, so haben wir es nicht weiter untersucht.

Das Filtrat vom Silbernitrat und Baryt-Niederschlag wurde wieder mit Phosphowolframsäure gefällt. Die freie Basenlösung, die durch Zerlegung des phosphowolframsauren Niederschlages durch Baryt erhalten wurde, lieferte nach dem Zusatz von wenig Pikrinsäure zuerst eine kleine Menge Argininpikrat. Bei weiterem Zusatz von Pikrinsäure schieden sich 3,5_g Kanirinpikrat aus, es bestand aus hellgelben Prismen mit dem Schmelzpunkt 188° (unkorr.) Es löste sich ziemlich schwer in Wasser. Aus heissem Alkohol schied es als rhombische Tafeln aus. Für die Analyse wurde das Pikrat im Vakuum bei 100° getrocknet

0,1246 _g Subst. gaben	19,7 ^{c.c.} N (13° 760 ^{mm})
0,1312 _g „	21,0 ^{c.c.} N (15° 766 ^{mm})
0,1526 _g „	0,1983 _g C O ₂ 0,0583 _g H ₂ O
0,4978 _g „	0,3762 _g Pikrinsäure
0,5098 _g „	0,3838 _g „ „

	C	H	N	Pikrin- säure
$C_6H_{14}N_2O_2 \cdot 2C_6H_3N_3O_7$ Ber.	35,76	3,31	18,54	75,83
	35,45	4,25	18,57	75,53
Gef.	—	—	18,89	75,48

Das salzsaure Kanirin bildete farblose, rhombische Prismen, mit dem Schmelzpunkt 204—207° (unkorr.). Es war hygroskopisch. Die wässrige Lösung des salzsauren Kanirins hatte kein Drehungsvermögen. Das Platinchloriddoppelsalz bestand aus tief rotbraunen Prismen, welche in Wasser ziemlich leicht, in Alkohol schwerer löslich waren. Im Kapillarrohr erhitzt, zersetzte es sich bei 227° (unkorr.). Für die Analyse wurde das Salz im Vakuum bei 100° getrocknet.

0,1444_g Subst, gaben 0,0504_g Pt

	Pt
$C_6H_{14}N_2O_2 \cdot H_2PtCl_6$ Ber.	35,00
Gef.	34,90

Ferner wurde versucht, den Methylester des Kanirins darzustellen, indem das salzsaure Salz in absolutem Methylalkohol verteilt und trockenes Salzsäuregas bis zur Sättigung eingeleitet wurde.

Nach dem Verdampfen des Methylalkohols schieden sich farblose Kristalle aus, welche einmal aus heissem Alkohol durch Zusatz von Aether umkristallisiert, im Vakuum bei 100° getrocknet und analysiert wurden.

0,1780 _g Subst. gaben	19,7 ^{c.c.} <i>N</i> (21° 760 ^{mm})
0,1710 _g „	0,2004 _g <i>C O</i> ₂ 0,1444 _g <i>H</i> ₂ <i>O</i>
0,2216 _g „	0,2804 _g <i>Ag Cl</i>

		<i>C</i>	<i>H</i>	<i>N</i>	<i>Cl</i>
$C_6 H_{14} N_2 O_2 \cdot 2 H Cl$	Ber.	32,90	7,30	12,70	32,40
$C_6 H_{13} N_2 O_2 (C H_3) 2 H Cl$	Ber.	36,08	7,72	12,02	30,47
	Gef.	31,96	9,38	12,60	31,28

Die Analyse weicht erheblich von dem salzsauren Methylester des Kanirins ab. Sie stimmt vielmehr mit dem salzsauren Salz des unveränderten Kanirins. Die kleine Differenz ist den hygroskopischen Eigenschaften des Salzes zuzuschreiben.

Das Salz zersetzte sich bei 207° ohne lebhaftes Schaümen. Die Kristallform war auch mit salzsaurem Kanirin identisch. Aus diesem Salze liess sich auch das ursprüngliche Pikrat gewinnen, welches den Schmelzpunkt 188° (unkorr.) hatte. Aus oben erwähnten Beobachtungen kann man schliessen, dass das Kanirin keinen Methylester bildet. Es kann deshalb nicht eine inaktive Isomerie des Lysins sein, obgleich das letztere auch dieselbe empirische Formel hat. Die Konstitution des Kanirins soll später aufgeklärt werden. Das Filtrat des phosphowölframsauren Niederschlags lieferte, nach der Estermethode verarbeitet, 0,14_g Alanin, 0,19_g Leucin und 0,11_g Tyrosin.

Der Tannin-Niederschlag, den wir aus wässrigem Krabbenextrakt durch unmittelbaren Zusatz von Tannin erhielten, lieferte nach dem Zerlegen mit Baryt ungefähr 1_g Argininpikrat. Analyse des Argininpikrats:

0,1134 _g Subst. gaben	24,7 ^{c.c.} <i>N</i> (21° 760 ^{mm})
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		<i>N</i>
$C_6 H_{14} N_2 O_2 \cdot C_6 H_3 N_3 O_7$	Ber.	24,32
	Gef.	24,79

Aus 2 kg frischem Krabbenfleisch wurden isoliert:

Argininpikrat	3,0 _g
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Tryptophan	vorhanden
Kanirinpikrat	3,5
Tyrosin	0,11
Alanin	0,14
Leucin	0,19
Histidin	wenig
Kreatin	„
Purinbasen	nicht vorhanden
Lysin	nicht vorhanden

Wie man sieht, weichen unsere Ergebnisse erheblich von denen ACKERMANN'S und KUTSCHERS ab. was der Verschiedenheit der untersuchten Krabbenart, deren verschiedenem Alter und anderen Bedingungen zuzuschreiben ist.

Das Tryptophan ist von uns zum ersten Male in frischem Fleisch nachgewiesen worden. Das Kanirin kommt auch im Taiffleisch vor.

IV. Tai (*Pagrus major*).

Von U. SUZUKI und Y. OKUDA.

63 kleine Taifische lieferten 3200_g Fleisch (von Kopf, Haut, Knochen und inneren Organen befreit) und daraus 7250^{cc} wässerigen Extrakt. Die quantitative Bestimmung des Extraktes gab folgendes Resultat:

	In 100 ^{cc} Extrakt	In 100 Teilen Trockensubstanz des Extraktes
Trockensubstanz	2,35	100,00
Gesamtstickstoff	0,27	11,54
Eiweiss „	0,112	4,770
Basen „	0,125	3,324
Ammoniak „	Spur	—

In 100 Teilen frischem Fleisch

Lösliche Substanz	5,324
Löslicher Stickstoff	0,611
Löslicher Eiweisstickstoff	0,253
Basenstickstoff	0,285

Der gesamte Extrakt wurde nun mit 20%iger Tanninlösung versetzt, vom entstandenen Niederschlag abfiltriert. Das Filtrat wurde nach dem Entfernen des Tannins durch Baryt und des überschüssigen Baryts durch Schwefelsäure, im Vakuum stark eingedampft und mit Alkohol versetzt. Es schieden sich dabei 5,6_g Kreatin aus, welches aus heissem Wasser umkristallisiert, bei 100° getrocknet und analysiert wurde.

0,1430_g Subst. gaben 38,0^{c.c.} N (8° 762^{mm})
 0,1457_g „ „ 0,1962_g C O₂ 0,0898_g H₂ O

		C	H	N
$C_4 H_9 N_3 O_2$	Ber.	36,64	6,87	32,06
	Gef.	36,72	6,91	32,15

Aus heissem Wasser umkristallisiert, scheidet es sich als grosse farblose glänzende Prismen aus. Bei 100° getrocknet, verliert es Kristallwasser. Die wässrige Lösung reagiert neutral. Mit verdünnter Schwefelsäure gekocht, wird es in Kreatinin verwandelt. Die Mutterlauge des Kreatins wurde mit Phosphowolframsäure gefällt.

A. DER PHOSPHOWOLFRAMSÄURE-NIEDERSCHLAG.

Man zerlegte den Niederschlag in gewöhnlicher Weise mit Baryt. Die alkalische Lösung, die freie Basen enthielt, wurde mit Kohlensäure gesättigt und mit Quecksilberchlorid gefällt.

a). Der Quecksilberchloridniederschlag lieferte, nach dem Entfernen des Quecksilbers durch Schwefelwasserstoff, 2,5_g Kreatininpikrat.

0,1926_g Subst. gaben 38,9^{c.c.} N (75° 769^{mm})
 0,1720_g „ 0,2239_g C O₂ 0,0461_g H₂ O

		C	H	N
$C_4 H_7 N_3 O$ $C_6 H_3 N_3 O_7$	Ber.	35,08	2,92	24,54
	Gef.	35,50	2,97	24,64

Das Pikrat schmolz bei 214° (unkorr.). Die wässrige Lösung gab eine rote Färbung bei Zusatz von verdünnter Alkali.

Im Filtrate des Kreatininpikrats war noch ein wenig Histidin vorhanden,

was durch Pauly'sche Reaktion nachgewiesen wurde. Isoliert haben wir es nicht.

b). Das Filtrat vom Quecksilberchloridniederschlag des Kreatinins wurde mittels Schwefelwasserstoff vom Quecksilber befreit, und mit Silbernitrat und Baryt gefällt. Dieser Niederschlag lieferte 0,75_g Kreatininpikrat.

Das Filtrat des Silbernitrat- und Baryt-Niederschlags wurde wieder mit Phosphowolframsäure gefällt. Aus diesem Niederschlag erhielt man 5,1_g Kaliumpikrat und von der Mutterlauge desselben 1,5_g Kanirinpikrat.

Analyse des Kaliumpikrats:

0,1698 _g Subst. gaben	22,4 ^{c.c.} <i>N</i> (12° 760 ^{mm})
0,1081 _g „	14,7 ^{c.c.} <i>N</i> (14,5° 758 ^{mm})
0,4969 _g „	0,4260 _g Pikrinsäure und 0,073 _g Kalium

	<i>N</i>	<i>K</i>	Pikrin- säure
<i>K C</i> ₆ <i>H</i> ₂ <i>N</i> ₃ <i>O</i> ₇ Ber.	15,73	14,28	85,72
Gef.	15,66	14,69	85,74
	15,90		

Analyse des Kanirinpikrats:

0,1331 _g Subst. gaben	0,1680 _g <i>C O</i> ₂ 0,0548 _g <i>H</i> ₂ <i>O</i>
0,3062 _g „	0,2344 _g Pikrinsäure

	<i>C</i>	<i>H</i>	Pikrin- säure
<i>C</i> ₆ <i>H</i> ₁₄ <i>N</i> ₂ <i>O</i> ₂ <i>C</i> ₆ <i>H</i> ₃ <i>N</i> ₃ <i>O</i> ₇ Ber.	35,76	3,31	75,83
Gef.	34,41	4,58	76,55

Das Kanirinpikrat schmolz bei 188° (unkorr.). Es war mit dem Kanirinpikrat aus Krabbenextrakt vollkommen identisch.

Das Platinchloriddoppelsalz des Kanirins.

0,1267_g Subst verloren bei 100° getrocknet 0,0043_g *H*₂ *O*

0,1142_g wasserfreie Substanz gaben 0,0400_g *Pt*

	<i>H</i> ₂ <i>O</i>	<i>Pt</i> als wasser- freies Salz
<i>C</i> ₆ <i>H</i> ₁₄ <i>N</i> ₂ <i>O</i> ₂ · <i>H</i> ₂ <i>Pt Cl</i> ₆ + <i>H</i> ₂ <i>O</i> Ber.	2,90	35,00
Gef.	3,39	35,02

Das Platinchloriddoppelsalz bestand aus rötlich gelben Prismen mit dem Schmelzpunkt 229° (unkorr.).

B. DAS FILTRAT VOM PHOSPHOWOLFRAMSÄURE-NIEDERSCHLAG.

Das Filtrat vom Phosphowolframsäure Niederschlag lieferte, nach der Estermethode verarbeitet, 0,14_g Alanin und 0,25_g Tyrosin. Analysiert haben wir die beiden Aminosäuren nicht.

C. TANNIN-NIEDERSCHLAG.

Der wässrige Extrakt des Fleisches gab eine reichliche Fällung mit Tannin. Beim Verreiben dieses Tannin-Niederschlags mit 2%iger Schwefelsäure wurde ein Teil desselben gelöst. Die vom unlöslichen Rückstand abfiltrierte Flüssigkeit wurde nun durch Baryt von Tannin und Schwefelsäure befreit und nach dem Entfernen des überschüssigen Baryts durch Schwefelsäure mit Tierkohle entfärbt und stark eingedampft.

Nach dem Erkalten schieden sich 1,5_g Kreatin aus.

Aus 3200_g frischem Fleisch wurden isoliert :

Kreatin	7,1 _g
Kreatininpikrat	3,25
Histidin	vorhanden
Kanirin	1,50
Alanin	0,14
Tyrosin	0,25

V. Hamaguri (*Cytheria meretrix*. L.).

Von U. SUZUKI und S. ŌDAKE.

17806_g lebende Hamaguri lieferten 3265_g Fleisch, nebst 8311_g Schale und 5480_g Wasser.

Das Fleisch wurde dreimal mit warmem Wasser (50°) extrahiert. Die vereinigten Auszüge betrugen 9250^{c.c.} und der Rückstand 814_g.

In 100 Teilen frischem Fleisch :

Trockensubstanz	45,86	
Darunter { Im wässrigen Extrakt	16,21	
{ Im unlöslichen Rückstand	29,65	
	In 100 Teilen	In 100 Teilen
	trockenem Fleisch :	Gesamtstickstoff :
Gesamtstickstoff	1,90	100,00
Gesamtstickstoff im wässrigen Extrakt	0,58	30,00
Darunter { Eiweis-stickstoff	0,23	12,10
{ Basen- „	0,21	11,10
{ Stickstoff in anderer Form	0,13	6,80
Gesamtphospor	0,17	

Aus der Analyse sieht man, dass ungefähr 70% des Gesamtstickstoffes im Fleisch aus wasserunlöslichen Eiweisstoffen besteht. Vom wasserlöslichen Stickstoff bestehen *ca.* 12% aus Eiweisstoffen.

Zur Isolierung der Basen wurden 8500^{cc} Extrakt (aus 3007_g frischem Fleisch = 1380_g Trockensubstanz) mit verdünnter Essigsäure schwach angesäuert und mit Tannin gefällt. Das Filtrat vom Tanninniederschlag wurde durch Bleiessig von Tannin und anderen Verunreinigungen befreit, und nach dem Entfernen des Bleies durch Schwefelsäure mit Schwefelsäure angesäuert und mit Phosphowolframsäure gefällt.

A. PHOSPHOWOLFRAMSÄURE-NIEDERSCHLAG.

Der Niederschlag wurde durch Baryt zerlegt. Während der Verarbeitung entwickelte sich Ammoniak und Trimethylamin, welche wahrscheinlich durch Zersetzung des Betains entstanden waren.

Die aus dem phosphowolframsauren Niederschlag dargestellte alkalische Lösung, die freie Basen enthielt, wurde mit Kohlensäure gesättigt und mit Quecksilberchlorid gefällt. Aus diesem Niederschlag erhielt man Histidin in kleiner Menge. Zur Analyse genügte es nicht. Das Filtrat vom Quecksilberchlorid-Niederschlag wurde durch Schwefelwasserstoff vom Quecksilber befreit und bei niederem Druck eingedampft, um den Schwefelwasserstoff vollständig auszutreiben, darauf mit Silbernitrat versetzt, um die vorhandene Salzsäure zu entfernen. Das Filtrat des Silberchlorids wurde nun mit überschüssigem Silbernitrat und Baryt versetzt. Aus diesem Niederschlag

wurde 2,1_g Arginindipikrat isoliert.

0,1546 _g Subst. gaben	0,1934 _g C O ₂	0,0536 _g H ₂ O
0,1500 _g „	29,9 ^{c.c.} N (24° 761 ^{mm})	
0,3998 _g „	0,2840 _g Pikrinsäure	

	C	H	N	Pikrin- säure
$C_6 H_{14} N_4 O_2 \cdot 2 C_6 H_3 N_3 O_7$ Ber.	34,18	3,17	22,15	72,47
Gef.	34,12	3,85	22,38	71,04

Aus heissem Wasser umkrystallisiert scheidet sich das Pikrat zuerst als ein Öl aus, nach dem Erkalten verwandelt es sich zu kleinen gelben Nadeln. Es löst sich leicht in warmem Wasser und Alkohol. In Aether und Petroläther ist es unlöslich. Im Kapillarrohr erhitzt sintert es bei ca. 160° und zersetzt sich bei 180—185° (unkorr.) unter Schäumen.

Da das aus Eiweisstoffen durch Spaltung mit Säuren erhaltene Arginin nur Monopikrat bildet, kann es nicht mit unserem Präparat identisch sein. Es handelt sich vielleicht um eine Isomerie desselben.

Aus dem Pikrate wurde das salzsaure Methylester des Arginins dargestellt. Es hat auch ein etwas anderes Verhalten als das Methylestersalz des Arginins aus Eiweiss zeigt. Die aus heissem Methylalkohol durch Zusatz von Aethylalkohol und Aether langsam ausgeschiedenen Krystalle bestanden aus scharf zugespitzten Nadeln, welche in Wasser und Methylalkohol sehr leicht, in Aethylalkohol etwas weniger, und in Aether und Petroläther fast unlöslich waren. Es schmolz bei 184° (unkorr.) unter lebhaftem Schäumen, während das Methylestersalz des Eiweiss-Arginins in demselben Bade bei 194° (unkorr.) schmolz.

Das Filtrat vom Silbernitrat und Baryt-Niederschlag bestand hauptsächlich aus Betain. Wurde das Filtrat durch Salzsäure vom Silber und durch Schwefelsäure vom Baryt befreit, und mit Phosphowalframsäure gefällt so entstand ein weisser flockiger Niederschlag, welcher nach Zerlegung mit Baryt farblose glänzende Krystalle von Betain in reichlicher Menge lieferte. Sie wurden abgesaugt, mit absolutem Alkohol und Aether gewaschen und über Schwefelsäure getrocknet. Die Ausbeute betrug 4,4_g. Zur Reinigung löst man die Krystalle in heissem Methylealkohol und setzt etwas Aether zu. Nach einiger Zeit scheidet sich das Betain als farblose

glänzende Tafeln aus, welche mit Aethylalkohol und Aether gewaschen werden.

Analyse des Betäins (im Vakuum bei 100° C getrocknet)

0,1526_g Subst. gaben 0,2913_g C O₂ 0,1386_g H₂ O
 0,1319_g „ 13,8^{c.c.} N (22° 758^{mm})

	C	H	N
C ₅ H ₁₁ N O ₂ . Ber.	51,19	9,40	11,97
Gef.	50,86	9,86	11,82

Das freie Betain bestand aus sehr hygroskopischen zerfliessenden Krystallen von einem angenehmen süssen Geschmack. Es löste sich in Wasser und Methylalkohol leicht, in Aethylalkohol etwas schwerer. In Aether und Petroläther war es fast unlöslich. Die wässerige Lösung reagierte schwach alkalisch, gab keine Biuret- oder Diazo-reaktion, keine rote Färbung mit Pikrinsäure und Alkali, und keine Fällung mit Nessler'schem Reagenz; mit Kupferhydroxyd erwärmt, gab es kein Kupfersalz. Im Kapillarrohr über 250° erhitzt, verkohlte es ohne zu Schmelzen. Es bildete zwei Nitrate, d.h. B₂ H N O₃ und B, H N O₃.

a). (C₅ H₁₁ N O₂)₂ . H N O₃ . Um dieses Salz darzustellen, wurde die freie Base mit einer berechneten Menge Salpetersäure versetzt (auf 1_g Betain ungefähr 10^{c.c.} normale Salpetersäure) und langsam fast bis zum Trocknen eingedampft. Beim Verreiben mit absolutem Alkohol schied sich das Salz vollständig aus, welches abgesaugt, mit absolutem Alkohol und Aether gewaschen wurde. Es wurde nochmals aus heissem absolutem Alkohol umkrystallisiert, im Vakuum bei 100° getrocknet und analysiert.

0,1445_g Subst. gaben 18,0^{c.c.} N (24° 762^{mm})
 9,1405_g „ 17,7^{c.c.} N (21° 753^{mm})
 0,1517_g „ 0,2233_g C O₂ 0,1120_g H₂ O
 0,2240_g „ 0,2835_g Nitronnitrat = 0,04765_g H N O₃

	C	H	N	H N O ₃
(C ₅ H ₁₁ N _k O ₂ .) H N O ₃ Ber.	40,40	7,74	14,14	21,21
Gef.	40,15	8,20	{ 14,01 14,17	21,26

Aus heissem Alkohol scheidet sich das Nitrat durch Zusatz von Aether

als farblose Prismen aus, die meistens sternförmig sich zusammen gruppieren. Es ist etwas hygroskopisch. In Wasser und Methylalkohol ist es leicht, in Aethylalkohol etwas schwerer und in Aether fast unlöslich. Im Kapillarrohr erhitzt, schrumpft es allmählich bei 215° und zersetzt sich bei $220-221^{\circ}$ (unkorr.) unter Gasentwicklung.

b). $C_5 H_{11} N O_2 \cdot H N O_3$. Will man dieses Salz darstellen, muss man etwas mehr als die berechnete Menge Salpetersäure zugeben. Bei langsamem Eindampfen und Versetzen mit absolutem Alkohol krystallisiert das Nitrat aus. Zur Reinigung wird das Salz in heissem absolutem Alkohol gelöst und durch Zusatz von Aether ausgeschieden.

Für die Analyse wurde das Salz im Vakuum bei 100° getrocknet.

0,1372_g Subst. gaben 18,7^{o.c.} N ($22,5^{\circ}$ 756^{mm})
 0,1320_g „ 0,274_g Nitronnitrat = 0,04603_g $H N O_3$

		N	$H N O_3$
$(C_5 H_{11} N O_2) H N O_3$	Ber.	15,55	35,00
	Gef.	15,32	34,87

Aus heissem Alkohol scheidet sich das Salz durch Zusatz von Aether als grosse farblose Tafeln aus. Es ist etwas hygroskopisch. Es schmilzt bei 120° zu einem Oel.

c). Das Pikrat. Sowohl aus $B_2 \cdot H N O_3$ als auch aus $B \cdot H N O_3$ erhält man ein und dasselbe Pikrat. Man löst das Nitrat ($B_2 H N O_3$) in wenig Wasser und gibt soviel Natronlauge zu, bis die Salpetersäure genau neutralisiert wird und versetzt mit Pikrinsäure in kleinem Überschuss. Das dabei aussgeschiedene Pikrat wurde einmal aus heissem Wasser umkrystallisiert, im Vakuum bei 100° getrocknet und analysiert.

0,1546_g Subst. gaben 0,2160_g C O_2 0,0596_g $H_2 O$
 0,1574_g „ 22,4^{o.c.} N (20° 760^{mm})
 0,4208_g „ 0,2810_g Pikrinsäure

		C	H	N	Pikrin- säure
$C_5 H_{11} N O_2 \cdot C_6 H_3 N_3 O_7$	Ber.	38,15	4,05	16,19	66,19
	Gef.	38,10	4,28	16,28	66,78

Das Pikrat besteht aus hellgelben Prismen und schmilzt bei 180—181° (unkorr.) zu einem Oel.

Aus $B \cdot H N O_3$ erhielt man auch dasselbe Pikrat mit dem Schmelzpunkt 180—181°. Die Analyse gab folgendes Resultat:—

0,1515_g Subst. gaben 22,3^{c.c.} N (25° 746^{mm})

		N
$C_5 H_{11} N O_2 \cdot C_6 H_3 N_3 O_7$	Ber.	16,19
	Gef.	16,11

Aus oben erwähnten Beobachtungen kann man schliessen, dass die Base Betain war. Ferner haben wir unser Präparat mit reinem Betain (Merck) verglichen. In süssem Geschmack, Schmelzpunkt, Krystallformen etc waren die beiden vollständig identisch.

Aus 17806_g frischen Cythereamuscheln = 1380_g trockenem Fleisch wurden isoliert:—

Argininpikrat	2,10 _g
Histidin	Vorhanden
Betain	4,40
Trimethylamin und Ammoniak	Vorhanden

VI. Austern.

Von U. SUZUKI, K. YOSHIMURA und Y. TANAKA.

Die lebenden Austern wurden von den Schalen getrennt und das Fleisch dreimal mit warmem Wasser extrahiert.

Die quantitative Analyse gab folgendes Resultat:—

	In 100 Teilen frischem Fleisch:	In 100 Teilen Trockensubstanz:
Wasser	92,16	
Trockensubstanz	7,87	100,00
Asche	3,29	41,96
Gesamtstickstoff	0,523	6,68
Eiweiss „	0,374	4,77

Wasserlöslicher Stickstoff		0,224	2,86	
Darunter	{	Eiweiss „	0,075	0,96
		Basen „	0,074	0,35
		Ammonia „	0,028	0,36
		Stickstoff in anderer Form	0,094	1,20

Gesamtstickstoff als 100.

Eiweisstickstoff	71,48	
Wasserlöslicher Stickstoff	42,86	
Darunter {	Eiweiss „	14,34
	Basen „	5,24
	Ammoniak „	5,39
	Stickstoff in anderer Form	17,89

Wie die Analyse zeigt, ist das Austernfleisch auffallend reich an Asche und Ammoniak. Das letztere ist ohne Zweifel schon zum Teil im lebendigen Fleisch vorhanden, teils wird es vielleicht aus Betain während der Extraktion gespalten. Ausser Ammoniak haben wir etwas Trimethylamin nachgewiesen.

Zur Isolierung der stickstoffhaltigen Substanzen wurde der wässrige Extrakt mit Tannin gefällt. Das Filtrat davon wurde durch Bleiessig von Tannin befreit; nachdem das überschüssige Blei durch Schwefelsäure entfernt war, wurde es mit Phosphowolframsäure gefällt.

A. DER PHOSPHOWOLFRAMSÄURE NIEDERSCHLAG.

Die aus diesem Niederschlag in bekannter Weise dargestellte Lösung, die freie Basen enthielt, wurde

- 1) mit Quecksilberchlorid gefällt, und das Filtrat
- 2) mit Silbernitrat und Baryt gefällt.
- 3) Das Filtrat vom Silbernitrat und Baryt-Niederschlag wurde nochmals mit Phosphowolframsäure gefällt.

Aus Fraktion (1) und (2) wurde keine Base in genügender Menge erhalten. Aus Fraktion (3) wurde 18^g fast reines Betain gewonnen. Die Analyse des freien Betains gab folgendes Resultat:—

0,4160_g Subst. gaben 0,05097_g N (nach Kjeldahl)

		<i>N</i>
$C_5 H_{11} N O_2$	Ber.	11,97
	Gef.	12,25

Das Platinchloriddoppelsalz des Betäins zersetzt sich bei 237—238° (unkorr.) unter Schäumen und Verkohlen.

0,2980 _g Subst. gaben	0,0810 _g <i>Pt</i> = 27,18% <i>Pt</i>
0,1800 _g „ „	0,0490 _g „ = 27,22%
0,1195 _g „ „	0,0328 _g „ = 27,03%

Diese Zahlen stimmen mit der Formel $(C_5 H_{11} N O_2)_2 H_2 Pt Cl_6 + 4 H_2 O$. Als wasserfreies Salz berechnet, muss es 30,29% sein. Das aus reinem Betain (Merck) dargestellte Platindoppelsalz gab auch unter derselben Bedingung 27,02% *Pt*. Es wurden ferner das Pikrat und die Nitrate dargestellt. In jeder Beziehung waren sie mit denen des reinen Betäins identisch.

B. DAS FILTRAT VOM PHOSPHOWOLFRAMSÄURE-NIEDERSCHLAG.

Das Filtrat wurde nach dem Entfernen der Phosphowolframsäure und Schwefelsäure durch Baryt bei vermindertem Druck stark eingedampft und mit Alkohol versetzt. Nach einiger Zeit schieden sich glänzende farblose Prismen in reichlicher Menge aus. Die Ausbeute derselben betrug 15,5_g. Nach der Umkrystallisation aus wenig Wasser zeigten sie die charakteristischen Krystallformen des Taurins.

Die Stickstoffbestimmung gab folgendes Resultat:—

0,3664 _g Subst. gaben	0,0412 _g <i>N</i> (nach Kjeldahl)
----------------------------------	--

		<i>N</i>
$C_2 H_7 N S O_3$	Ber.	11,20
	Gef.	11,24

Beim Glühen mit Glühgemisch hinterliess das Taurin eine Asche, die in verdünnter Salzsäure gelöst und mit Chlorbarium versetzt, einen weissen Niederschlag von Bariumsulfat gab.

Ferner war unser Präparat mit reinem Taurin (Merck) vollständig identisch.

Aus *ca.* 30 Kg. frischen Austern (mit Schalen) wurden isoliert :—

Betaïn	18,0 _g
Taurin	15,5 _g
Kreatin	?
Trimethylamin } Ammoniak }	Vorhanden

Tabelle der Extraktivstoffe.

	Auf 2 Kilo frisches Material berechnet.					
	Sardinen.	Maguro (Thynnus Thunnus).	Tai (Pagrus Major).	Krabben	Hamaguri (Cytherea Meretrix).	Austern*
Arginin				1,22	0,34	
Histidin	3,77	4,88	Vorhanden	Vorhanden	Vorhanden	
Imidazoläthylamin.. ..		0,52				
Carnosin	1,85					
Kanirin ($C_6H_{14}N_2O_2$) .			0,94	0,88		
Tryptophan				Vorhanden		
Leucin	0,23			0,19		
Alanin			0,09	0,14		
Tyrosin	1,70		0,16	0,11		
Kreatin		6,40	4,44	Vorhanden		?
Kreatinin.. .. .	0,63	Vorhanden	0,68			
Betaïn					2,86	18,0
Taurin						15,0
Trimethylamin u. Ammoniak.					Vorhanden.	Vorhanden.

* Aus *ca.* 30 Kg. frischen Austern (mit Schalen).

Quantitative Determination of Creatine, Creatinine and Monoamino-acids in certain Fishes, Mollusca and Crustacea.

BY

Y. Okuda.

I. Creatine and Creatinine.

For the determination of creatine and creatinine, the flesh freed from bones, heads, fins, scales, and internal organs¹ was chopped and extracted with water at 50—55° for one hour. The residue was treated twice more in the same way. The whole extract was now boiled for a short time to remove most of the proteins by coagulation and filtered. The filtrate was evaporated under diminished pressure to a small volume, and was divided into two portions. One portion of it served directly for the determination of creatinine after FOLIN's colorimetric method, while the other portion was previously boiled for two hours with nearly 4 per cent. sulphuric acid, to convert the creatine present into creatinine, and after removing the sulphuric acid by means of barium hydroxide, it was subjected to the determination after FOLIN. From the difference of these two determinations we can calculate the quantity of creatine originally present in the flesh, 1 mg creatinine being equivalent to 1.16 mg creatine. The results obtained were as follows :—

1. Of clams the whole body was used.

Name.	In 100 parts of fresh substance.			In 100 parts of dry matter.	
	Water g	Creatine g	Creatinine g	Creatine g	Creatinine g
Bonito (<i>Gymnonsarda affinis</i> Cantor)	72,165	0,649	0,134	2,011	0,481
Tunny fish (<i>Thunnus Schlegeli</i> Steind)	72,402	0,497	0,064	1,800	0,232
“Katsnobushi” (Steamed dried bonito)	14,808	0,453	0,660	0,531	0,775
Salmon (<i>Oncorhynchus tshawytscha</i> Walbaum)... ..	63,300	0,560	0,067	1,525	0,182
Snapper (<i>Pagrus major</i>)	77,340	0,754	0,070	3,327	0,308
Carp (<i>Cyprinus carpio</i> L.).. ..	79,160	0,421	0,077	2,020	0,369
Shark	79,800	0,655	0,134	3,242	0,663
Lobster (<i>Palinurus japonicus</i> Gray) ..	79,920	Trace ?	Trace ?		
Crab (<i>Neptunus pelagicus</i> M-Edw) ..	84,500	Trace ?	Trace ?		
Cuttle-fish (<i>Sepia esculenta</i> Hoyle) ..	81,699	Trace	Trace		
“Kakisurume” (chopped and dried cuttle-fish)... ..	27,570	Trace	Trace		
Clam (<i>Cytherea meretrix</i> L.)	90,490	Trace	Trace		

The materials used for the determination were very fresh, except the salted flesh of salmon.

We see from the above result that all of the examined fishes contained comparatively much creatine and creatinine,² on the contrary, in mollusca and crustacea the existence of these two compounds was doubtful, at least, they must be present only in traces. In fresh fish we found generally more creatine than creatinine, while in dried bonito the reverse was observed. It is therefore possible that a part of creatine is transformed into creatinine during the preparation of the food.

It may be mentioned here that the water extract of clam flesh gives only a slight yellowish red coloration instantly after addition of picric acid and soda after FOLIN, thus showing that only a trace of creatinine is present in it, but after standing for many hours at room temperature, it

2. VAN HOOGENHUYZE and H. HERPLOEGH found per kilogramm flesh of ox, sheep, pig and horse 4.4, 5.1, 4.5 and 3.8_g creatine respectively. (Zeitschr. f. physiol. Chemie., 1905, 46, 432.)

assumes a dark red color. After some tests we found that the glycogen, originally present in the extract is gradually acted upon by diastatic ferments of the clam itself, and the sugar thus formed imparts this red coloration. The presence of diastatic ferment in the clam is easily shown in the usual way.

II. Monoamino-acids.

For the determination of monoamino-acids SÖRENSEN'S formol titration method was adopted. Of course, this method does not hold good for some monoamino-acids, but in the case of fish flesh, the quantity of the aminoacids being very little, the method of VAN SLYKE can not conveniently be applied.

I have made also some preliminary tests and found that the presence of organic bases, like arginine, lysine, histidine etc. more or less affects the result of the formol method, so it is better to remove these bases previously. But the presence of creatine has apparently no effect upon this determination.

150_g minced fresh flesh, free from bones, heads, fins, scales and internal organs was extracted in a similar way as mentioned above and the aqueous extract was boiled and slightly acidified with acetic acid to remove coagulable proteins, filtered, neutralized and evaporated at low pressure into a small volume, acidified with sulphuric acid and precipitated with phosphotungstic acid in the usual way. The filtrate of the phosphotungstic precipitate, after the removal of the phosphotungstic and sulphuric acid by means of barium hydroxide, was evaporated, in neutral reaction, again to a small volume and titrated according to the usual formol method. Thus the following result was obtained:

Substance.	Water.	N of monoamino-acids in g.	
		In 100 _g fresh flesh.	In 100 _g dried flesh.
Carp I.	76,609	0,022	0,094
Carp II.	76,789	0,024	0,103
Tunny	73,516	0,011	0,041
Bonito	69,371	0,022	0,072
Porgy	76,787	0,016	0,069
Crussian carp	81,998	0,035	0,194
Spiny lobster	75,975	0,146	0,608
Cuttle fish	81,671	0,089	0,485

Remarks.—Tunny, bonito, porgy and cuttle fish applied to the above determination were fresh. Sping lobster, crussian carp and carp I were analysed immediately after having been killed. Carp II was analysed after standing for 50 hours after death at room temperature (12° C). The increase of amino-acids after that time was very insignificant.

We see from the above results that the contents of monoamino-acids are generally very little in fish, while mollusca and crustacea contain a little more.

III. On different Forms of Proteins in the Flesh of Fish.

For this purpose, the flesh was extracted³ with water, alcohol, *Na Cl*, and *KOH*, respectively and the quantity of total and albuminoid nitrogen in each extract was determined according to KJELDAHL's method.

Flesh	Solvent.	In 100 _g fresh flesh.		In 100 _g dry flesh.		Sum of each N as 100.	
		Total N	Prot. N	Total N	Prot. N	Total N	Prot. N
1. Crussian carp. (<i>Carassius auratus</i> L).	<i>H₂ O</i>	0,746	0,476	4,144	2,644	17,171	13,583
	0,2% <i>K O H</i>	2,003	1,793	11,127	9,960	46,077	51,125
	70% Alcohol	0,386	0,174	2,144	0,966	8,899	4,961
	10% <i>NaCl</i>	1,212	1,064	6,732	5,910	27,881	30,339
2. Carp. (<i>Cyprinus carpio</i> L).	<i>H₂ O</i>	0,479	0,326	2,182	1,485	11,129	10,326
	0,2% <i>K O H</i>	1,715	1,341	7,812	6,108	39,809	43,477
	70% Alcohol	0,492	0,240	2,241	1,093	11,420	7,602
	10% <i>NaCl</i>	1,622	1,250	7,388	5,694	37,649	39,605

3. 10_g fresh flesh was extracted with 100_{cc}. of solvent for 24 hours at 10°C.

Flesh	Solvent	In 100 _g fresh flesh		In 100 _g dry flesh		Sum of each <i>N</i> as 100.	
		Total <i>N</i>	Prot. <i>N</i>	Total <i>N</i>	Prot. <i>N</i>	Total <i>N</i>	Prot. <i>N</i>
3. Spiny lobster (<i>Palinurus japonicus</i> Gray).	H ₂ O	1,600	0,736	6,659	3,063	23,808	22,816
	0,2% KOH	2,138	1,212	8,898	5,044	31,813	37,572
	70% Alcohol	0,934	0,119	3,887	0,495	13,898	3,689
	10% NaCl	2,048	1,158	8,544	4,819	30,474	35,898
4. Cuttlefish (<i>Sepia esculenta</i> Hoyle)							
	H ₂ O	0,932	0,351	5,085	1,915	19,984	—
	0,2% KOH	1,775	1,223	9,684	6,673	38,059	—
	70% Alcohol	0,602	—	3,285	—	12,908	—
	10% NaCl	1,354	—	7,387	—	29,032	—

The amount of proteins extracted by alkali was generally much greater than that extracted by other solvents. The proteins soluble in 10% NaCl as globulins were also much, water soluble proteins as proteose and albumine not much and the proteins as prolamins very little.

IV. Form of Nitrogen in some Marine Animals.

The analytical results are shown in the following table. :—

	Carp. I. <i>Cyprinus carpio</i> . In 100 _g In 100 _g of fresh dry flesh. flesh. g g	Carp. II. <i>Cyprinus carpio</i> . In 100 _g In 100 _g of fresh dry flesh. flesh. g g	Bonito. <i>Gymnonsarda</i> <i>affinis</i> . In 100 _g In 100 _g of fresh dry flesh. flesh. g g	Porgy. <i>Pagrus major</i> In 100 _g In 100 _g of fresh dry flesh. flesh. g g	Crussian Carp. <i>Carassius</i> <i>auratus</i> . In 100 _g In 100 _g of fresh dry flesh. flesh. g g	Tunny. <i>Thunnus</i> <i>Schlegelii</i> . In 100 _g In 100 _g of fresh dry flesh. flesh. g g	Spiny lobster. <i>Palinurus</i> <i>japonicus</i> . In 100 _g In 100 _g of fresh dry flesh. flesh. g g	Cuttle fish. <i>Sepia esculenta</i> . In 100 _g In 100 _g of fresh dry flesh. flesh. g g
Water ⁴	76,609 —	76,789 —	69,371 —	76,787 —	81,998 —	73,516 —	75,975 —	81,671 —
Dry matter	23,391 100,000	25,211 100,000	30,629 100,000	23,213 100,000	18,002 100,000	26,484 100,000	24,025 100,000	18,329 100,000
Total N	2,608 11,149	2,590 11,057	4,479 14,621	3,121 13,445	2,655 14,746	3,549 13,397	3,558 14,808	2,496 13,619
Alb. N	2,107 9,007	1,972 8,495	3,799 12,401	2,685 11,584	2,285 12,691	— —	2,637 10,975	2,209 12,051
Non-alb. N	0,501 2,142	0,618 2,562	0,680 2,220	0,432 1,861	0,370 2,055	— —	0,921 3,833	0,287 1,568
Warm water soluble N	0,884 3,779	— —	1,628 5,314	1,025 4,419	1,039 5,771	1,530 5,776	1,586 6,601	0,919 5,013
Of which Protein N ..	0,431 1,843	— —	0,948 3,094	0,593 2,555	0,456 2,533	0,812 3,065	0,621 2,585	0,479 2,613
Organic base N	0,226 0,966	0,262 1,129	0,189 0,617	0,088 1,379	0,177 0,983	0,285 1,076	0,610 2,539	0,201 1,096
Monocamino N	0,022 0,094	0,024 0,103	0,022 0,072	0,016 0,069	0,035 0,194	0,011 0,041	0,146 0,608	0,089 0,495
Ammonium N	Trace	Trace	Trace	Trace	Trace	Trace	Trace	Trace
Reaction of flesh ..	Amphi- chromatic	Very faintly acidic	Acidic	Faintly acidic	Amphi- chromatic	Acidic	Amphi- chromatic	Amphi- chromatic
Water ⁵	79,160 —		72,165 —	77,340 —		72,402 —	79,920 —	81,699 —
Creatine.....	0,421 2,020		0,649 2,011	0,754 3,327		0,467 1,800	Trace?	Trace
Creatinine.....	0,077 0,369		0,134 0,481	0,070 0,308		0,064 0,232	Trace?	Trace
Creatine N	0,135 0,648		0,208 0,645	0,242 0,767		0,159 0,577		
Creatinine N	0,029 0,137		0,050 0,179	0,026 0,115		0,024 0,086		

4. Determined in September, 1910.

5. Determined in February, 1911.

Summary.

1. All of the examined kinds of fish contained comparatively much creatine and creatinine, but the flesh of mollusca only a trace, in the flesh of crustacea the existence of these compounds was doubtful. The quantity of creatine was generally much more than that of creatinine in all fresh fishes.

2. In all marine animals examined the quantity of organic base nitrogen is much more than that of monoamino-acid nitrogen, and the amount of the latter is generally very little in fish, but somewhat much in lobster and cuttle fish.

3. Most of the proteins are soluble in dilute alkali solution, the proteins soluble in 10% *NaCl* were also much, this fact must be taken in consideration in the preservation of fish.

The experiments have been made by the writer under the direction of Professor Dr. U. SUZUKI, and it is my pleasant duty to thank him for his kind advice given during the progress of the work.

Über die chemische Zusammensetzung des "Salzbreies" von Bonito ("Shiokara").

VON

U. Suzuki, C. Yoneyama und S. Ōdake.

Zur Bereitung des "Salzbreies" wird der Magen und Darmkanal des Bonitos vom innern Inhalt befreit, gut gewaschen, fein zerhackt und mit viel Kochsalz vermischt, so dass ein dicker Brei entsteht. Die Leber wird auch manchmal dazu gemengt. Man lässt nun den Brei wochenlang bei Zimmertemperatur stehen und rührt öfters um. Es tritt dabei allmählich die Reifung ein, ein eigentümlicher Geruch und Geschmack entwickelt sich, und von vielen Leuten, besonders von Sakékennern wird der Artikel als Delikatesse mit Vorliebe genossen. Die an der Reifung des Breies teilnehmenden Mikroben sind bis jetzt nicht untersucht, und die chemischen Vorgänge, die während des Reifeprozesses vor sich gehen, sind auch nicht näher erforscht. Nur vermutet man, dass sie den bei der "Shoyu"-bereitung auftretenden ziemlich ähnlich sind. Durch Einwirkung von Mikroben und Enzymen werden verschiedene Stoffe, besonders Eiweissstoffe, allmählich gelöst und abgebaut, unter Bildung von Peptonen und Aminosäuren, die zum Teil weiter desamidiert, oxidiert oder reduziert werden. Es entstehen dabei verschiedene Säuren, Alkohole, Amine u. s. w. Die Zusammensetzung des Breies ist deshalb sehr kompliziert. Es kommen bei verschiedenen Reifestadien verschiedene Stoffe zum Vorschein. Wir beschränken uns vorläufig mit der Untersuchung der stickstoffhaltigen Bestandteile des käuflichen, gereiften Breies.

Das von uns untersuchte Material war aus Odawara bezogen. Es war graurötlich braun gefärbt und reagierte ziemlich stark sauer.

Die quantitative Bestimmung gab folgendes Resultat:

	In 100 Teilen frischem Brei
Wasser	65,13
Trockensubstanz	34,87

	In 100 Teilen Trockensubstanz
Organische Stoffe	30,06
Asche	69,94
Chlor	29,80
(als NaCl berechnet)	49,18

	In 100 _g frischem Brei.	Gesamt-N. als 100.
Gesamtstickstoff	1,735	100,0
Eiweiss- „	0,472	27,2
Basen- „	0,447	25,7
Ammoniak- „	0,131	7,6
Stickstoff in anderer Form	0,685	39,5

Zur Isolierung der stickstoffhaltigen Stoffe wurden 4 Kilo Brei ausgepresst. Der Rückstand wurde dreimal mit warmem Wasser (40—50°) extrahiert. Die vereinigten Auszüge, die schwach sauer reagierten, betrugen rund 9 Liter. Sie wurden mit 20%iger Tanninlösung gefällt. Der Tannin-Niederschlag (B) wurde abgesaugt und mit Wasser gewaschen. Das Filtrat vom Tannin-Niederschlag wurde mit verdünnter Natronlauge versetzt, bis es schwach alkalisch reagierte. Es entstand dabei eine flockige Fällung (C) in reichlicher Menge. Man saugte davon ab, und setzte dem Filtrat viel Baryt zu, um das Tannin zu entfernen, saugte wieder ab, und nach dem Entfernen des Baryts mittels Schwefelsäure dampfte man bei niederem Druck stark ein. Es schieden sich dabei Tyrosin, Leucin und anorganische Salze aus.

Aus heissem Wasser umkrystallisiert erhielt man zuerst 3_g Tyrosin und von der Mutterlauge desselben 2,1_g Leucin. Die beiden Aminosäuren wurden nochmals für sich umkrystallisiert und analysiert.

Tyrosin:

0,1604_g Subst. gaben 10,7^{c.c} N (16°. 760.^{mm})

		<i>N</i>
$C_9 H_{11} N O_3$	Ber.	7,70
	Gef.	7,78

Leucin :

0,1719_g Subst. gaben 15,7^{c.c} *N* (14°. 758^{mm})

		<i>N</i>
$C_6 H_{13} N O_2$	Ber.	10,07
	Gef.	10,71

Die Mutterlauge von Leucin und Tyrosin wurde mit Schwefelsäure angesäuert und mit Phosphowolframsäure gefällt.

H. DER PHOSPHOWOLFRAMSÄURE-NIEDERSCHLAG.

Die aus diesem Niederschlag dargestellte alkalische Flüssigkeit, die freie Basen enthielt, lieferte nach starkem Einengen im Vakuum keine Krystalle ; sie wurde mit Kohlensäure gesättigt und mit Quecksilberchlorid gefällt.

a) Der Quecksilberchlorid-Niederschlag wurde mit Schwefelwasserstoff zerlegt, im Vakuum eingedampft und mit Pikrinsäure erwärmt. Nach dem Erkalten schied sich 8,5_g fast reines Lysinpikrat aus, welches aus heissem Wasser umkrystallisiert und analysiert wurde.

0,1414_g Subst. gaben 22,0^{c.c} *N* (13°. 766^{mm})
 0,1491_g „ „ 0,2094_g *C O*₂ 0,0612_g *H*₂ *O*.
 0,4149_g „ „ 0,2533_g Pikrinsäure.

			<i>C</i>	<i>H</i>	<i>N</i>	Pikrin- säure
$C_6 H_{14} N_2 O_2$	$C_6 H_3 N_3 O_7$	Ber.	38,40	4,53	18,67	61,07
		Gef.	38,34	4,56	18,53	61,05

Im Kapillarrohr erhitzt zersetzte sich das Pikrat gegen 247° (unkorr). Das Platinchloriddoppelsalz des Lysins bestand aus hygroskopischen, goldgelben, langen Prismen. Es schmolz bei 205° (unkorr). Für die Analyse wurde es im Vakuum bei 100° getrocknet.

0,3014_g Subst. gaben 0,1055_g Pt.

			Pt.
$C_6 H_{14} N_2 O_2$	$H_2 Pt Cl_6$	Ber.	35,00
		Gef.	35,00

b) Das Filtrat vom Quecksilberchlorid-Niederschlag wurde nach dem Entfernen des Quecksilbers durch Schwefelwasserstoff und der Salzsäure durch Silbernitrat mit einem Überschuss von Silbernitrat und Baryt versetzt. Der braune Niederschlag lieferte 1,3_g Lysin-pikrat.

Die Analyse des vereinigten Salzes gab folgendes Resultat:—

0,1319_g Subst. gaben 21,3^{c.c.} N (20°. 760^{mm})

			N
$C_6 H_{14} N_2 O_2$	$C_6 H_3 N_3 O_7$	Ber.	18,67
		Gef.	18,56

c) Das Filtrat vom Silbernitrat- und Baryt-Niederschlag wurde in bekannter Weise mit Phosphowolframsäure gefällt. Aus diesem Niederschlag erhielt man wieder 6_g Lysin-pikrat.

0,1374_g Subst. gaben 22,3^{c.c.} N. (21°. 763^{mm})

0,1526_g „ „ 0,2160_g C O₂ 0,0672_g H₂ O.

			C	H	N
$C_6 H_{14} N_2 O_2$	$C_6 H_3 N_3 O_7$	Ber.	38,40	4,53	18,67
		Gef.	38,60	4,89	18,55

B. DER TANNIN-NIEDERSCHLAG (TRYPTOPHAN).

Der vom wässrigen Extrakt des Salzbreies durch Zusatz von Tannin erhaltene Niederschlag wurde mit 3%iger Schwefelsäure wiederholt verrieben. Ein Teil ging dabei in Lösung. Man filtrierte nun vom unlöslichen Rückstand ab, und setzte dem Filtrate viel Baryt zu, um damit Tannin und Schwefelsäure wegzuschaffen. Das vom dabei entstandenen Niederschlag abgesaugte Filtrat wurde mit Schwefelsäure angesäuert und mit Phosphowolframsäure gefällt.

a) Durch Zerlegung des phosphowolframsauren Niederschlags wurde eine Flüssigkeit erhalten, welche schöne Tryptophanreaktion gab. Wird diese Flüssigkeit mit Essigsäure angesäuert und mit einigen Tropfen Bromwasser versetzt, so entsteht eine rot violette Färbung. Beim Schütteln mit Amylalkohol geht der Farbstoff in das letztere Reagenz über. Um das Tryptophan zu isolieren, wurde die Flüssigkeit mit so viel Schwefelsäure versetzt, bis sie 5% der Säure enthielt, und mit Hopkin'schem Reagenz gefällt. Es entstand dabei eine weisse flockige Fällung, die abgesaugt, mit 5%iger Schwefelsäure gewaschen und mit Schwefelwasserstoff zerlegt wurde. Beim Eindampfen des Filtrats im Vakuum schied sich ein Teil des Tryptophans krystallinisch aus. Die Hauptmasse blieb jedoch amorph, so dass das gereinigte Tryptophan nicht zur Analyse ausreichte. Aus dem Filtrate vom Quecksilbersulfat-Niederschlag des Tryptophans wurde eine Base als pikrinsaures Salz isoliert. Dies genügte auch zur weiteren Untersuchung nicht.

b) Das Filtrat des phosphowolframsauren Niederschlages lieferte, nach der Estermethode verarbeitet, eine kleine Menge Leucin.

C. DER TANNIN- UND NATRONLAUGE-NIEDERSCHLAG.

Wie oben erwähnt, liefert das Filtrat vom Tannin-Niederschlag (B) durch Zusatz von verdünnter Natronlauge, wieder eine reichliche Fällung, die eine nicht unbeträchtliche Menge Basen enthielt. Um die Basen zu isolieren, wurde der Niederschlag mit 5%iger Schwefelsäure verrieben, wobei ein grosser Teil in Lösung ging. Die braune Flüssigkeit wurde nun mit einem Überschuss von Baryt versetzt, vom dabei entstandenen Niederschlag abgesaugt, mit Schwefelsäure angesäuert und mit Phosphowolframsäure gefällt. Nach Zerlegung des phosphowolframsauren Niederschlages in bekannter Weise erhielt man eine alkalische Flüssigkeit, die freie Basen enthielt.

Diese Flüssigkeit wurde nun mit Kohlensäure gesättigt und mit Quecksilberchlorid gefällt.

a) Der Quecksilberchlorid-Niederschlag (Histidin).

Aus diesem Niederschlag erhielt man eine alkalische Flüssigkeit, die

sowohl starke Pauly'sche Reaktion, wie auch Biuretreaktion beim Erwärmen gab. Bei Zusatz von Pikrinsäure wurde 0,5_g Histidinpikrat gewonnen, welches aus heissem Wasser umkrystallisiert, im Vakuum bei 100° getrocknet und analysiert wurde.

0,1430_g Subst. gaben 27,8^{c.c.} N (24°. 759^{mm})
 0,1517_g Subst. gaben 0,2034_g C O₂ 0,0457_g H₂ O.

				C	H	N
C H ₉ N ₃ O ₂ . C ₆ H ₃ N ₃ O ₇	Ber.			37,50	3,13	21,91
				Gef. 37,56	3,35	21,77

Die Analyse stimmt also mit dem Histidinpikrat überein. Der Schmelzpunkt war jedoch viel höher als bei gewöhnlichem Histidinpikrat, welches aus Eiweiss durch Spaltung mit Säuren dargestellt wird. Im Kapillarrohr erhitzt, wurde es gegen 200° braun und zersetzte sich gegen 210° (unkorr.) unter Schäumen. Es handelt sich wahrscheinlich um eine Isomerie des Histidins. Wegen Mangel an Material konnten wir das optische Verhalten nicht untersuchen.

b) Das Filtrat vom Quecksilberchlorid Niederschlag des Histidins wurde mit Silbernitrat und Baryt gefällt. Aus diesem Niederschlag isolierte man eine Base als pikrinsaures Salz, welches 1,5_g betrug. Das Pikrat bestand aus rotbraunen blättrigen Krystallen mit dem Schmelzpunkt 225° (unkorr.). Die Analyse gab folgendes Resultat:

0,1171_g Subst. gaben 24,2^{c.c.} N (20°. 700^{mm})
 0,1372_g „ „ 0,1715_g C O₂ 0,0496_g H₂ O.
 0,2504_g „ „ 0,1919_g Pikrinsäure

				C	H	N	Pikrin- säure
C ₆ H ₁₄ N ₄ O ₂ . (C ₆ H ₃ N ₃ O ₇) ₂	Ber.			34,18	3,16	22,15	72,47
				Gef. 34,09	4,02	23,64	76,63

Die Analyse stimmt also mit dem Arginindipikrat; nur ist der Gehalt an Stickstoff und Pikrinsäure etwas höher.

c) Das Filtrat vom Silbernitrat- und Baryt-Niederschlag (b) wurde wieder mit Phosphowolframsäure gefällt. Der Niederschlag lieferte 2,34_g Lysinpikrat mit dem Zersetzungspunkt 245° (unkorr.)

D. DAS FILTRAT DES PHOSPHOWOLFRAMSAUREN NIEDERCHLAGES.

Das Filtrat vom Phosphowolframsäure-Niederschlag wurde nach der Estermethode verarbeitet, indem die Phosphowolframsäure und Schwefelsäure durch Baryt entfernt und der Überschuss vom Baryt mittels Schwefelsäure beseitigt und im Vakuum stark eingedampft wurde. Der zurückgebliebene Syrup wurde nun mit absolutem Alkohol versetzt, mit trockenem Salzsäuregas gesättigt und in bekannter Weise in die freien Estern der Aminosäuren verwandelt. Nach fraktionierter Destillation der Estern wurden die folgenden drei Fraktionen erhalten:—

	Temperatur	Estermenge.	Aminosäuren nach der Verseifung.
I.	bis 75° (20 ^{mm})	9,0 _g	3,0 _g
II.	75 — 100° („ „)	10,5 _g	7,8 _g
III.	über 100° („ „)	7,0 _g	6,0 _g

Fraktion I. bestand aus Alanin. Aus heissem Wasser umkrystallisiert, bildete es farblose Nadeln mit süßem Geschmack und zersetzte sich gegen 270°. Für die Analyse wurde es im Vakuum bei 100° getrocknet.

0,1553_g Subst. gaben 21,3^{c.c.} N (17° 760^{mm})
 0,1530_g „ „ 0,2248_g C O₂ 0,1040_g H₂ O.

		C	H	N
C ₃ H ₇ N O ₂	Ber.	40,45	7,87	15,88
	Gef.	40,07	7,55	16,03

Fraktion II. bestand auch hauptsächlich aus Alanin, nebst einer kleinen Menge Prolin.

Analyse des Alanins:—

0,1476_g Subst. gaben 20,4^{c.c.} N (21° 751^{mm})

		N
C ₃ H ₇ N O ₂	Ber.	15,88
	Gef.	15,62

Kupfersalz des Alanins:

0,2114_g Subst. gaben 0,0703_g CuO.

		<i>Cu</i>
$(C_3 H_6 N O_2)_2$	Ber.	26,22
	Gef.	26,57

Fraktion III. bestand zum grössten Teil aus Leucin, nebst Alanin und Prolin. Zweimal aus heissem Wasser umkrystallisiert, wurde das Leucin in ziemlich reinem Zustande erhalten. Es schmeckte schwach bitter und zersetzte sich gegen 280°.

Analyse des Leucins :

0,1467_g Subst. gaben 13,4^{c.c.} *N* (19°. 762^{mm})

		<i>N</i>
$C_6 H_{13} N O_2$	Ber.	10,07
	Gef.	10,56

Das Prolin wurde isoliert, indem die nach der Verseifung der Estern erhaltenen Aminosäuren mit heissem absoluten Alkohol extrahiert wurden. Der vereinigte alkoholische Extrakt wurde eingedampft und der Rückstand nochmals mit absolutem Alkohol extrahiert. Nach dem Verdampfen des Alkohols wurde das Prolin in bekannter Weise in das charakteristische Kupfersalz verwandelt, welches in heissem absoluten Alkohol löslich war. Die Ausbeute an Kupfersalz betrug 1,03_g. Für die Analyse wurde das gereinigte Salz im Vakuum bei 100° getrocknet.

0,1613_g Subst. gaben 13,8^{c.c.} *N* (22°. 752^{mm})

		<i>N</i>
$(C_5 H_8 N O_2)_2$	Ber.	9,60
	Gef.	9,59

Ferner wurde das Vorhandensein von Glutaminsäure im Filtrat des phosphowolframsauren Niederschlages durch ihren charakteristischen faden Geschmack ausser Zweifel gestellt. Wegen Mangel an Zeit haben wir diese Säure nicht isoliert.

Aus 4 Kilo Salzbrei vom Bonito wurden isoliert:—

1. Lysinpikrat. 18,14_g.

Chemical Studies on the Ripening of "Shiokara."

BY

Y. Okuda.

Although the isolation and identification of some nitrogenous compounds in "Shiokara" has been undertaken about two years ago by Prof. U. SUZUKI, YONEYAMA, and ŌDAKE in this laboratory, no chemical investigation about the ripening process of this interesting food material has yet been reported. So I have tried to contribute something on this line. I have observed that the autolysis and the action of microbes are two indispensable factors for the preparation of "Shiokara."¹ Some trials have also been made to isolate the enzymes which play an important rôle in this process, and finally, I have carried out some quantitative determinations to see the chemical changes at different stages of ripening.

I. Autolysis and the Action of Microbes.

1). To see whether autolysis is going on during the ripening process of "Shiokara," very fresh organs² of a bonito fish were minced with a meat-chopping machine, and rubbed with some quartz sand in a mortar. 40g of the paste thus prepared was divided into two equal parts and put in the flasks A and B. After adding 100 c.c. of water to each flask, A was boiled for a few minutes to destroy the enzymatic action. Both flasks were then shaken with enough toluol and a little chloroform, and kept for 4 days at ordinary temperature. No bacterial growth was observed during that

1. "Shiokara" made from the organs of bonito was used.
2. The stomach, intestines and pyloric cœcum.

time. The flask B was now boiled, and the contents of both flasks were then filtered and analysed with the following results:—

	A (boiled)	B (not boiled)
Total soluble nitrogen	1.697 %	1.895 %
Soluble albuminoid nitrogen	0.184 „	0.141 „
Non-albuminoid nitrogen	1.513 „	1.754 „

2). The fresh “Shiokara” two days after preparation was chopped and crushed in a mortar, 200g of the paste were divided into two equal parts, put in two flasks of 1 litre capacity and stoppered with cotton plugs. After adding 500 c.c. of water, one flask was boiled. To each flask was now added enough toluol and chloroform, and after keeping for 4 days at room temperature, the contents of both flasks were filtered and analysed.

	A. (boiled)	B. (not boiled)
Total soluble nitrogen	1.848 %	2.052 %
Soluble albuminoid nitrogen	0.056 „	0.038 „
Non-albuminoid „	1.792 „	2.014 „
Amino nitrogen (after formol method)	0.604 „	1.023 „

We see from the above two experiments that autolysis is going on in the fresh organs of bonito fish, and also in the freshly prepared “Shiokara.”

3). The microbes predominating in “Shiokara” seem to be quite different at different stages of its ripening. In three preparations, made in April and two months old, we found immense numbers of yeasts, bacilli and cocci, but only few moulds, while in a sample prepared early in October and about one and a half months old, were found numerous yeasts, the other microbes being relatively very few.

The isolation and identification of these microbes will be reported afterwards.

4). 120g of the “Shiokara”, which was two months old, were well crushed and equally divided into three Erlenmeyer's flasks containing each 100 c.c. of saturated sodium chloride solution and treated in the following way.

A. Control:—Not boiled, no antiseptics added.

B. Not boiled, toluol and chloroform added to prevent the bacterial growth, but not the enzymatic action.

C. Boiled and antiseptics added to prevent both bacterial and enzymatic action.

After keeping for ten days at 34-38°, they were boiled and filtered, and the filtrates were analysed with the following results:—

	A. (Control)	B. (not boiled but antiseptic added)	C. (boiled and antiseptic added)
Total soluble nitrogen	2.404 %	2.305 %	2.305 %
Soluble alb. nitrogen	0.049 „	0.090 „	0.184 „
Non-alb. nitrogen	2.355 „	2.215 „	2.121 „

The above experiment shows that both autolysis and the action of microbes are going on very slowly in the old preparations compared to fresh ones. The investigation of WEHMER³ on salted herring has shown that the action of microbes upon proteins is somewhat retarded in 5 per cent. common salt solution, but it does not entirely stop even in 30% solution. As the concentration of the salt in "Shiokara" usually is 15%, there is no doubt that the microbes can still play an important rôle on the ripening process, especially at the early stage of its preparation.

II. Enzymes in "Shiokara."

Trypsin, diastase and lipase were identified in the fresh organs of a bonito fish and also in the fresh preparations of "Shiokara." In the old preparation however, their action seems to be much retarded. This observation agrees well with the experiments mentioned above.

1). Fresh organs. The stomach, intestines and pyloric coecum of a fresh bonito were freed from their contents and rubbed with some quartz sand in a mortar, and filtered through the cloth filter. The faintly acid extract thus obtained has shown its peptonifying power upon milk and fibrin, either in the faintly acid reaction or after addition of 0.2% sodium carbonate. But

3. WEHMER. Abhandlungen des deutschen Seefischerei-Vereins, III, 1898, 1.

no action was observed in presence of 0.2% hydrochloric acid in the medium, thus the absence of pepsin is most probable.

The existence of diastase was shown by the saccharification of starch paste and glycogen in the neutral reaction.

For the detection of lipase, the minced and ground organ was extracted with a mixture of 90 parts of pure glycerine and 10 parts of 1% sodium carbonate, 10 c.c. of the mixture being used for 1g of the sample. The liquid was filtered through a piece of cloth and exactly neutralized. By the addition of some milk or olive oil to this extract, the increase of acidity due to the formation of fatty acids by the action of lipase upon neutral fats was observed. Of course some toluol and chloroform being added to prevent the bacterial growth.

2). "Shiokara" at different stages of ripening. The following observation was made with the samples collected at different stages of ripening:—

(a). "Shiokara," two days old.

Trypsin.	Present, active.
Diastase.	Do.
Lipase.	Present, but the action was very weak.

(b). "Shiokara," 40-50 days old.

Trypsin.	Present, but very weak.
Diastase.	No reaction.
Pepsin.	Do.

(c). "Shiokara," 50-60 days old.

Trypsin.	Very weak.
Lipase.	Do.
Diastase.	No reaction.

3). Isolation of enzymes. For this purpose, about 200g of the fresh sample, 3 days old, were finely minced and ground with some quartz sand in a mortar and macerated with a little distilled water. The liquid was strained through linen cloth, and after dialysing for about two hours to get rid of the greater part of the common salt, it was poured into a mixture of absolute alcohol and ether, the grayish white voluminous precipitate thus produced was then collected on a filter, washed with absolute alcohol and ether, and dried over sulphuric acid. The crude enzyme preparation obtained in

this way, when dissolved in a little water, has shown strong diastatic and tryptic action while that of lipase was very weak. When the solution of these crude enzymes was added to a solution of various amino-acids, no liberation of ammonia was observed, showing the absence of amidase.

The proteolytic enzyme, which acts in weak alkaline as well as in neutral or in faintly acid reaction, but not in a 0.2% hydrochloric acid solution, was also found by BLANCHARD⁴ in several fish and by ROAF⁵ in two crustacea.

III. Chemical Changes during the ripening Process.

1). The sample⁶ used for this determination was prepared on the 17th of June, 1911, and after 3, 6, 12, 25, and 40 days respectively a portion was taken for analysis. Thus the following results were obtained:—

IN 100 PARTS OF FRESH SAMPLES.

Date of analysis	3	6	12	25	40	days after preparation.
Water	64.95	64.78	64.58	64.25	63.99	
Dry matter.. .. .	35.05	35.22	35.42	35.75	36.01	
Total N	1.98	2.04	2.05	—	2.07	
Alb. N.. .. .	0.35	0.35	0.28	0.26	0.14	
Ether extract	1.83	1.81	1.81	1.71	1.74	
Soluble matter	27.25	28.45	29.24	31.10	31.14	
Non-alb. N.. .. .	1.63	1.69	1.83	1.98	2.01	
Ammonium N	0.15	0.15	0.25	0.18	0.13	
Organic base N	0.84	0.72	0.69	0.63	0.64	
Other N	0.74	0.80	1.09	1.17	1.43	
Total acid (as lactic) .. .	1.43	1.42	0.97	0.96	0.98	
NaCl (calculated from total chlorine) ..	17.31	—	17.34	—	17.71	

4. BLANCHARD, Jahresbericht für Tierchemie, 13, 1883-orig. Compt. rend. 96, 1241.
5. ROAF, Jahresber. f. Tierchemie, 36, 1906-orig. Biochem. Journal. 1, 390-97.
6. This sample contained the stomach, intestines, pyloric coecum and very little liver.

IN 100 PARTS OF DRY MATTER.

Total N	5.64	5.79	5.79	—	5.76	
Alb. N.. .. .	1.00	1.00	0.80	0.71	0.38	
Ether extract	5.21	5.13	5.10	4.77	4.84	
Soluble matter	77.74	80.76	82.53	86.99	86.49	
Non-alb. N	4.64	4.79	5.18	5.54	5.57	
Ammonium N	0.43	0.43	0.70	0.51	0.36	
Organic base N	2.39	2.04	1.97	1.77	1.79	
Other N	2.12	2.26	3.09	3.26	3.98	
Total acid	4.07	4.02	2.73	2.67	2.72	
Common salt	49.37	—	48.95	—	49.17	

2). The second sample⁷ was prepared on the 3rd of Oct. 1911, and after 1, 14, and 53 days respectively, a portion was taken for analysis:—

	In 100 parts of fresh sample.			In 100 parts of dry matter.			days after preparation.
	1	14	53	1	14	53	
Date of analysis							
Water.. .. .	64.39	63.00	60.33	0.0	0.0	0.0	
Dry matter	35.61	36.99	39.67	100.0	100.0	100.0	
Total N	2.29	—	—	6.42	—	—	
Total acid	0.95	1.05	1.14	2.65	2.85	2.88	
Ether extract	6.84	6.89	—	19.19	18.61	—	
Soluble matter	25.61	27.14	28.90	71.92	73.27	72.86	
Non-alb. N	1.59	1.85	2.07	4.45	4.99	5.21	
Ammonium N	0.10	0.12	0.14	0.28	0.32	0.35	
Organic base N.. .. .	0.81	0.73	0.71	0.27	1.96	1.79	
Creatinine N	0.01	trace	trace	2.02	trace	trace	
Creatine N.. .. .	0.02	trace	trace	0.04	trace	trace	
Xanthine base N	—	0.06	0.03	—	0.16	0.08	
Other N	0.66	1.00	1.21	1.86	2.71	3.05	
Sodium chloride	13.51	13.94	—	37.94	37.62	—	

7. This sample contained more liver than the former one.

The results of the above two analyses may be summarized as follows :—

	(1)	(2)
Soluble organic matter.	Gradually increased.	Do.
Alb. N	„ decreased	—
Non-alb. N	„ increased	Do.
Ammonium N	Increased at first and decreased henceforward.	Gradually increased
Monoamino N	Gradually increased	Do.
Organic base N	„ decreased	Do.
Creatine N	—	Gradually decreased
Creatinine N	—	Do.
Xanthin base N	—	Do.
Ether extract	Somewhat decreased	Do.
Total acid	Decreased	Somewhat increased

Thus the results of two analyses resemble each other in general respects, only the contradictory results were observed with ammonia and with total acid. This may be due to the differences of materials and temperature during the experiments.

3). I will add here some qualitative tests made about the distillates obtained by the steam distillation of two Shiokara preparations.

In the distillate. 10 days after preparation. 61 days after preparation.

Alcohol	(+) ^s (very little)	(—)
Aldehyde	(—)	(—)
Acetone	(—)	(—)
Indol	(—)	(—)
Phenol	(—)	(—)
Formic acid	(+) (trace)	(+) (distinct)

In the residue.

Lactic acid	(+) (distinct)
Succinic acid	(—)

In the water extract of the natural sample.

Tryptophan	(+)	(+)
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8. (+) indicates presence; (—) absence.

Summary of the Results.

1. Various samples, examined at different stages of ripening, gave all acid reaction chiefly due to lactic acid.

2. Autolysis is going on in the freshly prepared "Shiokara," and decreases gradually as the ripening process proceeds.

3. The enzymes found in "Shiokara" are diastase, lipase and trypsin. The last one acts not only in weak alkaline solution but even in neutral or in faintly acid reaction.

4. Microorganisms play also some important rôle during the ripening process.

5. Temperature has also great influence upon the action of enzymes and microbes.

6. During the ripening process, the increase of soluble matter, non-albuminous nitrogen, especially monoamino-nitrogen, and the decrease of protein, organic bases, creatinine, creatine, and purin bases were observed.

In conclusion I express my thanks to Profs. U. SUZUKI and S. MACHIDA for their kind advice given during the progress of this work.

On Koji Acid, a new organic Acid formed by *Aspergillus Oryzae*.

BY

T. Yabuta.

Dr. K. SAITO has recently isolated a new organic acid from *Aspergillus Oryzae*, which was grown on steamed rice. For this purpose he has extracted the finely powdered fungus mass with hot water, evaporated the extract into a syrup, which was repeatedly shaken with ether. On evaporating the ethereal solution he has obtained the crude acid, which was purified by recrystallization from hot water. It forms colourless needles, giving a strong red coloration with ferric chloride. The qualitative tests have shown that this acid is not identical with oxalic, succinic, citric, malic, tartaric or benzoic acid, which usually occur in the lower fungi. Judging from the melting point and from the characteristic reaction with ferric chloride, he supposed it to be identical with β -resorcylic-carbonic acid. But owing to the difficulty of obtaining the pure acid in large quantities, he did not investigate it any further, so I have tried to clear up the chemical nature of this acid. For this purpose I have prepared fairly large quantities of it as pure as possible, and confirmed the empirical formula to be $C_{12}H_{14}O_8$. From the copper salt, as well as from the acetyl derivative and benzoyl derivative, the presence of two carboxyl and four hydroxyl groups in the molecule has also been ascertained, so that the formula may be written as $C_{10}H_8(OH)_4(COOH)_2$. It is therefore quite different from β -resorcylic carbonic acid and so far as I know, the occurrence of such an acid in fungi has never been mentioned before. I have given the name "Koji acid" to this acid.

Isolation of Koji Acid.

For the isolation of koji acid in large quantities I have modified Dr. SAITO's method in the following way :

1). 150_g steamed rice are put in an ERLLENMEYER flask of 500_{c.c.} capacity, which is provided with cotton air filter, and after repeated sterilization in KOCH's steam sterilizer, it is inoculated with the spores of *Aspergillus oryzae* and kept at 30—35° for 2—3 weeks, being shaken from time to time. When the rice is fully covered with the myceliums and brown spores of the fungus, it is dried at low temperature, finely powdered, and extracted with ether in a large SOXHLET's extraction apparatus. During the extraction a part of koji acid crystallizes out as colourless needles from the ethereal solution, while a part remains in the solution. The ethereal solution is now evaporated to dryness, and treated with petroleum ether to remove the fatty matter. The crude acid thus obtained is dissolved in a little hot water, decolorized with animal charcoal (which should be free from trace of iron) and evaporated. About 50_g koji acid can be thus obtained from 15 kilo rice.

2). Finely powdered koji is extracted with boiling alcohol. The alcoholic extract is evaporated by reduced pressure nearly to dryness. The residue is dissolved in water and precipitated with basic lead acetate, avoiding an excess. The precipitate thus produced is filtered off and the filtrate therefrom is treated with sulphuretted hydrogen to remove the excess of lead and evaporated in vacuum to expel the sulphuretted hydrogen. The acid solution thus obtained is neutralized with caustic soda. By adding a copper sulphate solution to it, the light green copper salt of koji acid is precipitated at once, which is washed with water and decomposed with sulphuretted hydrogen. The filtrate of the copper sulphide which contains the free koji acid is evaporated to a small volume. After cooling the acid separates as colourless needles which may be purified by recrystallization.

The free acid crystallizes in colourless needles or prisms with no water of crystallization. It melts at 152° (not corr.) to a brownish liquid. The crystals have a bitter taste, while the aqueous solution is somewhat sour.

The purified acid is rather stable in the air. It is readily soluble in ethyl alcohol, methyl alcohol, water and acetone, less easily in pyridin, slightly in ether and chloroform, and quite insoluble in petroleum ether, ligroin, benzene and carbon tetrachloride. The aqueous solution has an acid reaction to congo red, phenol phthalein, rosolic acid, litmus and methyl orange. It expels carbonic acid gas from an alkaline carbonate or bicarbonate solution. The aqueous solution gives a strong wine-red coloration with ferric chloride, which may be recognized even in the dilution of 1/200,000. No reaction is, however, obtained with ferrous salts. The red coloration disappears on addition of diluted mineral acids and reappears by neutralization. As already mentioned, koji acid is precipitated from its aqueous solution by copper acetate or sulphate, the precipitation being complete, when the solution is previously neutralized with dilute alkali. The acid is not precipitated from its aqueous solution by aqueous or ammoniacal silver nitrate, neutral, basic or ammoniacal lead acetate, tannin, mercuric chloride, phosphotungstic and phosphomolybdic acid. It has no reaction upon alkaline diazo-benzolsulphonic acid, NESSLER'S reagent, MILLON'S reagent, FEHLING'S solution. The aqueous solution of the acid absorbs much bromine. Neither a methoxyl nor ethoxyl group is recognized by ZEISSEL'S method. When potassium permanganate is added to the alkaline or alkali carbonate solution of koji-acid, the latter is oxidized, oxalic acid being one of the oxidation products.

Koji acid leaves no ash after ignition, it contains no nitrogen, sulphur, phosphorus or halogen in its molecule.

Different samples of the acid, purified from various solvents, such as water, alcohol, acetone and ether, were dried in vacuum at 100°, and analysed with the following results:—

1.	0.1770 _g subst. gave	0.3264 _g C O ₂	0.0776 _g H ₂ O
2.	0.1810 _g „ „	0.3340 _g „	0.0840 _g „
3.	0.1374 _g „ „	0.2542 _g „	0.0626 _g „
4.	0.2240 _g „ „	0.4132 _g „	0.1000 _g „

$C_{12} H_{14} O_8$		% C.	% H	% O
	calc.	50.35	4.90	44.75
	found	1. 50.29	4.87	44.84
		2. 50.33	5.15	44.52
		3. 50.49	5.06	44.45
		4. 50.31	4.96	44.73

The Copper Salt.

The copper salt is easily obtained by adding copper acetate or copper sulphate to the aqueous solution of the acid, which is previously neutralized with caustic soda. The precipitate is collected on a filter and washed with hot water, until the filtrate gives no reaction for sulphuric acid.

The copper salt forms light green rhombic crystals, containing no water of crystallization. It is insoluble in water, alcohol, ether, benzene, petroleum ether and ligroin, but soluble in dilute mineral acids as well as in acetic acid. It dissolves also in dilute ammonia with blue coloration.

The analysis of the salt dried in vacuum at 100° gave the following result :—

1.	0.1179 _g subst. gave	0.1782 _g C O ₂	0.0395 _g H ₂ O
2.	0.1085 _g „ „	0.1644 _g „	0.0365 _g „
1.	0.5000 _g subst. gave	0.1160 _g Cu O	
2.	0.7824 _g „ „	0.1782 _g Cu O	

$C_{12} H_{12} O_8 Cu.$		% C	H	% Cu
	calc.	41.42	3.45	18.32
	found	1. 41.22	3.73	18.49
		2. 41.32	3.68	18.19

The Acetyl Derivative.

1). The acetyl derivative is obtained by boiling the acid with five times its weight of acetic anhydride in a flask connected with reverted cooler. After heating for 2 hours, it is evaporated under diminished pressure to remove the excess of acetic anhydride and the acetic acid formed by the reaction. The residue is recrystallized several times from hot alcohol.

2). In the previous method acetic anhydride may be mixed with half of its weight of anhydrous sodium acetate. The acetylated product can be easily separated from sodium acetate by means of ethyl acetate, owing to their different solubilities.

3). One part of the acid and two parts of acetyl chloride are put into a flask, provided with reverted cooler, and gently warmed on the water bath. The reaction takes place very violently with the evolution of hydrochloric acid gas. When the reaction is over, it is evaporated in vacuum to remove the excess of acetyl chloride and hydrochloric acid, and the residue is purified as mentioned above.

The acetyl compound crystallizes from the hot alcoholic solution as colourless needles, which melt at 102° to a brownish liquid. It is easily soluble in ethyl acetate, somewhat less in alcohol, and only sparingly in cold water. It dissolves, however, readily in hot water. The acetyl compound has a slightly acid reaction. It gives no precipitate with copper acetate, nor does it show any reaction with ferric chloride, but the above reactions are obtained when it is previously saponified with caustic alkali and neutralized with hydrochloric acid.

Analysis of the acetyl compound, dried in vacuum at 80° gave the following result:—

1.	0.1051 _g subst. gave	0.2030 _g C O ₂	0.0465 _g H ₂ O	
2.	0.1234 _g subst. „	0.2386 _g „	0.0552 _g „	
		% C	% H	% O
C ₁₂ H ₁₀ O ₄ (O. C H ₃ C O) ₄ . calc.		52.86	4.84	42.30
found 1.		52.67	4.92	42.41
2.		52.77	4.97	42.26

To give further proof that the acetyl compound has four acetyl radicals in one molecule, I have determined the acetyl value in the following way:

Into 2 ERLIENMEYER flasks (A and B) were put 1_g each of the sample and after dissolving in 50^{cc} water, one flask (A) was directly titrated with standard caustic soda solution, while the other flask (B) was previously saponified by adding 100^{cc} of standard caustic soda solution. After keeping

for 48 hours at room temperature, just so much standard sulphuric acid was added as to neutralize 100^{c.c.} caustic soda and then titrated with standard soda solution. The difference between the two titrations (A and B) expresses the quantity of standard soda solution, which was used to neutralize the acetic acid produced by the saponification of the acetyl compound. Thus I found :

		Na O H used			
		sample used	before saponification (A)	after saponification (B)	Difference (B—A)
$C_{12}H_{10}O_4 (O.CH_3CO)_4$	calc.	1.000	22.04	66.18	44.14
	found 1.	1.000	22.00	66.50	44.50
	2.	1.000	22.00	67.00	45.00

From these observations, it is very probable that the original acid has four hydroxyl groups in one molecule.

The Benzoyl Derivatives.

Two benzoyl derivatives, namely di- and tetra-benzoate corresponding to the formula $C_{12}H_{10}O_4(OH)_2(O.C_6H_5CO)_2$, respectively $C_{12}H_{10}O_4(O.C_6H_5CO)_4$, were obtained in the following way.

1). Dibenzoyl derivative.

1_g. finely powdered koji acid is suspended in 40^{c.c.} absolute ether, 10^{c.c.} benzoyl chloride is added to it and heated in a flask, connected with reverted cooler, for 2—3 hours. After cooling, the reaction product is collected on a filter and washed with petroleum ether to remove the adhering benzoyl chloride. The residue is then dissolved in a little alcohol. On addition of large quantities of cold water, the benzoyl compound separates at once as colourless needles, which may be further purified by repeating the same process.

The benzoyl compound melts at 137° to a brownish liquid. It dissolves only sparingly in cold water, but more easily in hot water. The solubility in alcohol is greater than in water. In ether it is slightly soluble. The benzoyl compound has scarcely any acid reaction. It gives no reaction with

copper acetate or with ferric chloride. After saponifying, however, it gives the above reaction.

Analysis of the benzoyl compound, dried in vacuum at 100° gave the following result:

1.	0.1256 _g	subst. gave	0.2874 _g	C O ₂	0.0530 _g	H ₂ O	
2.	0.0862 _g	„ „	0.1994 _g	„	0.0370 _g	„	
C ₁₂ H ₁₀ O ₄ (O H) ₂ (O. C ₆ H ₅ C O) ₂				% C	% H	% O	
			calc.	63.15	4.45	32.40	
			found	1.	63.13	4.69	32.18
			2.	63.08	4.76	32.16	

2). Tetra-benzoyl derivative.

1_g koji acid dissolved in 50^{cc} water is shaken with 10^{cc} benzoyl chloride and 50^{cc} caustic soda solution (15%), cooling with cold water, until the odour of benzoyl chloride vanishes. The benzoyl derivative separating from the solution is collected on a filter, washed with water and recrystallized from hot alcohol which was slightly acidified with hydrochloric acid. The crystals thus obtained are treated with water until the filtrate gives no reaction for chlorine. For further purification they are repeatedly recrystallized from hot alcohol.

The benzoyl derivative forms colorless plates which melt at 135° . It is hardly soluble in water but readily in alcohol or glacial acetic acid.

Analysis of the benzoyl derivative, dried in vacuum at 100° gave the following result:

0.1412 _g subst. gave		0.3530 _g C O ₂	0.0526 H ₂ O		
C ₁₂ H ₁₀ O ₄ (O. C ₆ H ₅ C O) ₄			% C	% H	% O
		calc.	68.37	4.28	27.35
		found	68.23	4.14	27.63

Different varieties of *Aspergillus oryzae* seem to produce different amounts of koji acid under same conditions. According to my observation, the varieties isolated from "Tamari-koji" produce more acid than those isolated from "Shoyu-" or "Sake-koji." Generally those varieties which liquefy gelatine more powerfully seem to produce more acid.

The acid is also produced by some other *Aspergillus* species, but not by *Penicillium* or *Mucor*, as the following table shows:—

<i>Asp.</i>	<i>oryzae</i>	+	<i>Penicillium glaucum</i> ..	—
„	<i>albus</i>	+	„ <i>glaurosus</i> ..	—
„	<i>candidus</i>	+	„ <i>olivaceum</i> ..	—
„	<i>Okazakii</i>	—	<i>Mucor amylomyces</i> ..	—
„	<i>melleus</i>	—	„ <i>racemosus</i> ..	—
„	<i>minimus</i>	—	„ <i>circineloides</i> ..	—
„	<i>fumigatus</i>	—	„ <i>spinosus</i>	—
„	<i>Wenti</i>	—	„ <i>mucedo</i>	—
„	<i>niger</i>	—	„ <i>stolonifer</i>	—
„	<i>ochraceus</i>	—	<i>Oidium lactis</i>	—
„	<i>glaucus</i>	—	<i>Botolytis cineria</i>	—
„	<i>luchuensis</i>	—	<i>Dematium pullulans</i> ..	—
„	<i>nidulans</i>	+	<i>Monilia candida</i>	—
„	<i>flavus</i>	—	„ <i>javanica</i>	—
„	<i>clavatus</i>	—	„ <i>variabilis</i>	—
„	<i>giganteus</i>	—	<i>Chalara mycoderma</i> ..	—
„	<i>varians</i>	—		

As the acid is also produced by the aspergillus when cultured in “koji extract,” it is easily detected by previously adding a few drops of ferric chloride to the culture medium.

Different culture media behave also differently upon the production of this acid. Thus, the latter is produced when the aspergillus has grown on certain cereals, such as maize, wheat, barley, rye, oats and Italian millet, but not on leguminous seeds, as peas, beans, horse beans, broad beans, soja beans, Indian beans, black beans, quail beans, etc. Among many root crops examined, sweet potato was the only one which produces this acid.

The antiseptic power of koji acid upon mould fungi and yeasts seems to be very weak, as Dr. SAITO has already reported. Certain fungi can even assimilate it as a nutrient. But the bacterial growth is generally stopped, when the nutritive medium contains more than 0.5% of this acid.

Über das Vorkommen von Nikotinsäure (m-Pyridin-carbonsäure) in der Reiskleie.

VON

U. Suzuki und S. Matsunaga.

Während wir uns mit der Untersuchung des Oryzanins in der Reiskleie beschäftigten, haben wir als ein Nebenprodukt Nikotinsäure gefunden. Da diese Säure unseres Wissens zum ersten Male im Pflanzenreich gefunden worden ist, so machen wir eine kurze Mitteilung darüber.

Zur Isolierung dieser Säure wurde die entfettete Reiskleie wiederholt mit 80-85%igem heissem Alkohol extrahiert. Der alkoholische Extrakt wurde stark eingedampft, um den grössten Teil des Alkohols auszutreiben. Der Rückstand wurde in wenig Wasser gelöst, abfiltriert und mit Aether geschüttelt, um die Fette und andere Verunreinigungen zu entfernen. Nach dem Austreiben des Aethers wurde die Flüssigkeit mit verdünnter Schwefelsäure versetzt, bis diese ungefähr 3% erreichte, und mit Phosphowolframsäure gefällt. Der Niederschlag wurde abgesaugt, mit wenig 3%iger Schwefelsäure gewaschen und in bekannter Weise mit Baryt zerlegt. Die vom Bariumphosphowolframat abfiltrierte und bei niederem Druck stark eingeeengte Flüssigkeit reagierte nach dem Entfernen des überschüssigen Baryts mittels Schwefelsäure gegen unsere Erwartung ziemlich stark sauer, und bei Zusatz von Pikrinsäure in kleinem Überschuss schied sich ein Pikrat aus, das in Wasser und in Alkohol ziemlich schwer löslich war. Es liess sich daher beim Erwärmen mit wenig Alkohol leicht von der Verunreinigung trennen. Aus heissem Wasser umkrystallisiert schied sich das Pikrat als hellgelblichweisse sandige Krystalle aus. Im Kapillarrohr erhitzt schmilzt es bei 214° (unkorr.) unter Zersetzung. Für die Analyse wurde das Salz im Vakuum bei 100° getrocknet.

Analyse des Pikrats.

1)	0.1602 _g Subst.	0.2376 _g C O ₂	0.0348 _g H ₂ O	
	0.1291 _g „	19.0 ^{c.c.} N (21.° 757 ^{mm})		
	0.1076 _g „	0.0705 _g Pikrinsäure		
2)	0.1511 _g „	0.2254 _g C O ₂	0.0350 H ₂ O	
	0.1342 _g „	18.2 ^{c.c.} N (12° 763.5 ^{mm})		
		C	H	N Pikrinsäure
	C ₆ H ₅ N O ₂ , C ₆ H ₃ N ₃ O ₇ Ber.	40.91	2.27	15.91 65.06
	Gef.	40.45	2.41	16.51 65.50
		40.68	2.57	16.19 —

Die Analyse stimmt also mit dem Nikotinsäurepikrat ziemlich gut.

Die Ausbeute an Pikrat betrug ca. 1 gr. aus 1 Kilo Kleie, was aber bei verschiedenen Kleiearten sehr schwankend sein kann.

Die freie Nikotinsäure wurde dargestellt, indem das Pikrat in Wasser gelöst, mit verdünnter Schwefelsäure angesäuert, und wiederholt ausgeäthert wurde, um die Pikrinsäure vollständig zu entfernen. Die wässrige Lösung wurde dann mittels Baryt von der Schwefelsäure befreit, und bei gelinder Wärme langsam eingedunstet. Es schieden sich dabei farblose, lange Nadeln aus, welche abgesaugt, mit wenig kaltem Wasser, Alkohol und Aether gewaschen wurden. Für die Analyse wurde die freie Säure nochmals aus Wasser umkrystallisiert, und im Vakuum bei 100° getrocknet.

Analyse der freien Säure.

0.1394 _g Subst.	0.2983 _g C O ₂	0.0542 _g H ₂ O	
0.0707 _g „	7.05 ^{c.c.} N (14° 764.5 ^{mm})		
	C	H	N
C ₆ H ₅ N O ₂ Ber.	58.54	4.07	11.38
Gef.	58.36	4.32	11.80

Die freie Säure schmilzt bei 228°-229°. Sie ist in kaltem Wasser und Alkohol etwas schwer, in heissem Wasser aber leichter löslich. In Aether und Petrolaether ist sie fast unlöslich. Die wässrige Lösung reagiert stark sauer. Aus verdünnter saurer Lösung wird sie durch Phosphowolframsäure gefällt.

Das Kupfersalz. Wird die wässrige Nikotinsäurelösung mit einer Kupferacetatlösung in kleinem Überschuss versetzt, so entsteht sofort ein hell

blau-grüner Niederschlag des Kupfersalzes, welches in Wasser, Alkohol und Aether schwer löslich ist. Unter dem Mikroskop beobachtet bildet das Kupfersalz rundliche Kugeln oder Sphaerokrystalle. Im Kapillarrohr erhitzt zersetzt es sich bis 300° nicht. Für die Analyse wurde es bei 100° getrocknet.

Analyse des Kupfersalzes.

0.1108 _g Subst.		0.0291 _g Cu O	
0.2231 _g „		17.7 ^{c.c.} N (20.°5 760 ^{mm})	
		N	Cu
(C ₆ H ₄ NO ₂) ₂ Cu.	Ber.	9.13	20.68
	Gef.	9.06	20.94

Das Platinchloriddoppelsalz. Es bestand aus weisslich gelben Krystallen.

0.1653_g Subst. gaben 0.0497_g Pt.

		Pt	
(C ₆ H ₅ NO ₂ H Cl) ₂ Pt Cl ₄	Ber.	29.72	
	Gef.	30.00	

Aus den oben erwähnten Beobachtungen kann man schliessen, dass die von uns isolierte Säure Nikotinsäure war.

Wir haben ferner unsere Säure mit der reinen Nikotinsäure, die wir von Prof. K. Ikeda im Chemischen Institut der hiesigen Universität bekommen haben, verglichen. Die beiden Präparate waren in jeder Beziehung vollkommen identisch.

Es sei hier erwähnt, dass eine Homolge der Nikotinsäure d. h. Picolin-carbonsäure vor einigen Jahren von O. SCHREINER⁽¹⁾ aus humusreichem Boden isoliert worden ist.

(1) O. SCHREINER u. E. C. SHOREY: U. S. Department of Agriculture, Bureau of Soils, Bulletin No. 53.

Über das Vorkommen von Adenin und Asparaginsäure in Maulbeerblättern.

VON

Z. Mimuroto.

Bekanntlich sind Maulbeerblätter ziemlich reich an Nicht-Eiweiss-Stickstoff, besonders in jungen Blättern. Nach der Analyse von O. Kellner macht er manchmal 25% des Gesamtstickstoffs aus, so ist zu vermuten, dass verschiedene stickstoffhaltige Verbindungen darin vorhanden sind. Vorläufig habe ich kleine Versuche angestellt, um diese Stoffe zu isolieren und ist es mir gelungen, das Vorhandensein von Adenin und Asparaginsäure festzustellen. Zu diesem Zwecke wurden 500_g lufttrockene und fein gepulverte Blätter mit 4 l Wasser bei 50-55° zwei Stunden digeriert, stark abgepresst. Der Rückstand wurde noch zweimal in derselben Weise behandelt. Die vereinigten Auszüge wurden mit Bleiessiglösung so lange versetzt, bis keine Fällung mehr entstand. Nach einiger Zeit wurde abfiltriert und das Filtrat mit 30%igem Quecksilberniträt versetzt. Als der grösste Teil der Acidität durch Natronlauge neutralisiert war, entstand eine weisse flockige Fällung in reichlicher Menge, die mit kaltem Wasser gewaschen, in wenig Wasser verteilt und durch Schwefelwasserstoff zerlegt wurde. Die vom Quecksilbersulfid abfiltrierte Flüssigkeit wurde bei niederem Druck stark eingedampft und unmittelbar mit Pikrinsäure erwärmt. Es schieden sich dabei feine gelbe Nadeln aus, welche abgesaugt und mit wenig kaltem Wasser gewaschen wurden. Die Ausbeute betrug 1,2_g.

Aus heissem Wasser umkrystallisiert, bildet das Pikrat lange, sich zusammen gruppierende Nadeln. Es löst sich in kaltem Wasser ziemlich schwer. Im Kapillarrohr erhitzt, zersetzt es sich bei ca. 289°.

Für die Analyse wurde es im Vakuum bei 100° getrocknet.

Analyse des Pikrats :

0,0456 _g Subst. gaben	0,0594 _g C O ₂	0,0124 _g H ₂ O
0,0446 _g „ „	12,2 ^{c.c.} N (19° 752 ^{mm})	
0,5000 _g „ „	0,3090 _g Pikrinsäure	

		C	H	N	Pikrin- säure
$C_5H_5N_5, C_6H_3N_3O_7$	Ber.	36.25	2.21	30.78	62.80
	Gef.	35.53	3.02	31.23	61.80

Um die freie Base darzustellen wurde das Pikrat in wenig Wasser gelöst, mit verdünnter Schwefelsäure angesäuert und wiederholt mit Aether geschüttelt, um die Pikrinsäure vollständig zu entfernen. Nach dem Entfernen der Schwefelsäure mittels Baryt, wurde die wässrige Lösung der Base bei gelinder Wärme fast bis zum Trocknen eingedampft. Es schieden sich dabei fast farblose kleine Krystalle aus, welche in kaltem Wasser ziemlich schwer, in heissem Wasser leichter löslich waren. In Mineralsäuren, Alkalien und Ammoniak lösten sie sich sehr leicht. Im Kapillarrohr erhitzt sublimierten sie ohne zu schmelzen. Sie gaben die WEIDEL'sche sowie die STRECKER'sche Reaktion nur schwach. Wird die wässrige Lösung mit einem Stückchen Zink und Salzsäure im Wasserbade erwärmt, so tritt eine vorübergehende, schöne Rosafärbung ein, welche durch Zusatz von Natronlauge rotbraun wird.

Alle diese Eigenschaften stimmten mit reinem Adenin überein. Zur Analyse genügte das Material nicht, daher beabsichtige ich diese Versuche nochmals mit grösseren Mengen zu wiederholen.

Die Mutterlauge des Adeninpikrats wurde nach der Entfernung der Pikrinsäure mit Schwefelsäure angesäuert und mit Phosphowolframsäure gefällt. Aus dem phosphowolframsauren Niederschlag habe ich eine peptonartige Substanz in kleiner Menge erhalten.

Das Filtrat vom phosphowolframsauren Niederschlag wurde in bekannter Weise nach der FISCHER'schen Estermethode verarbeitet. Nach Verseifung des freien Esters wurden 0,3_g Aspaarginsäure isoliert. Da die Menge derselben nicht zur Analyse ausreichte, habe ich unmittelbar das Kupfersalz dargestellt. Es bestand aus hellblauen Nadeln, mit dem Schmelzpunkt 224°

(unkorr.) Die Krystallformen, Schmelzpunkt und Löslichkeit u. s. w. stimmten mit reiner Asparaginsäure vollständig überein.

Analyse des Kupfersalzes:—

0.100_g Subst. gaben

0.0268_g *Cu O*

		<i>Cu O</i>
$C_4 H_5 N O_4 Cu + 4\frac{1}{2} H_2 O$	Ber.	26.8
	Gef.	26.2

Während der Verarbeitung des freien Esters habe ich die Entwicklung des Ammoniaks beobachtet. Daraus erscheint es sehr wahrscheinlich, dass das Asparagin, während der Behandlung mit Salzsäuregas in Asparaginsäure und Ammoniak gespalten war.

Im wässerigen Extrakt von Maulbeerblättern habe ich ferner ziemlich viel Glukose als Osazon isoliert.

A Contribution to the Study of the Chemical Composition of the Silkworm at different Stages of its Metamorphosis.

BY

R. Inouye.

The experiments have been carried out at the suggestion of Prof. U. SUZUKI, first with Mr. T. TAKENOUCI and then with Mr. K. C. BHARATKAR. In the course of analysis great assistance has been kindly given by S. ŌDAKE and S. IWAOKA.

During the metamorphosis of the silkworm, a great chemical change is supposed to take place in its body. Dr. O. KELLNER¹ investigated the feeding of the silkworm and found neither ammonia nor hippuric acid in the excrement of the silkworm, but uric acid was a chief constituent of it; the silkworm was not able to digest fibre, but non-albuminoid nitrogenous compounds could be digested in a small quantity; and the silkworm developed no nitrogen gas while living and also no volatile nitrogenous compounds. F. ANDERLINI² investigated the content of glycogen during metamorphosis and concluded that the pupa and the female moth contained it in the largest quantity.

E. BATAILLON³ studied the quantity of carbon dioxide gas exhaled on pupating and attributed the decrease of the gas production in the pupal stage to its condensing in the body.

LUCIANI and LO MONACO⁴ also studied the respiration of the silkworm. E. CONOREUR⁵ found the increase of glycogen when the silkworm changed

- 1). O. KELLNER: Landw. Versuchsstat. Bd. XXX, 1882, P. 59 and Bd. XXXIII, 1886, P. 35.
- 2). F. ANDERLINI: Atti del R. Institute Veneto di Scienze 5, 1291-1294.
- 3). E. BATAILLON: Compt. rend. 115, 61-64.
- 4). LUCIANI and LO MONACO: Arch. ital. de Biologie 23, 424.
- 5). E. CONOREUR: T. Chemie 1895, 25, 402.

into the pupa even without taking any nutrient, the content of fat being nearly constant; the latter, however, greatly decreases when the pupa becomes moth.

C. VANEY and F. MAIGNON¹ investigated the relative quantity of glucose, glycogen, fat and carbohydrate. Lately EMIL ABDERHALDEN and WOLFGANG WEICHARDT² studied the monoaminoacids which were formed by the total hydrolysis of silkworms and moths, and concluded that the moth is to be considered as silkworm minus cocoon, in other words, the silkworm, when changing into the pupa, produced on hydrolysis large quantities of glycocoll, alanine, and tyrosine while the moth produced them only in small quantity. The above is a general sketch concerning the chemistry of the silkworm. How the chemical composition of the silkworm is changed at each phase of its metamorphosis has not yet been minutely studied, as far as I know, and that investigation is the aim of the present analysis. Some preliminary notes, however, must first be made.

A. Preparation of the Sample.

Some 1000 silkworms of a spring-race, *Koishimaru*, which had been reared at the Tokyo Sericultural Institute, were collected at their maturity (on the 6th June, 1910), and some 200 of them, weighing 475_g, were at once killed by sulphuretted hydrogen gas, dried up on a water bath and ground into fine powder for analysis. The rest were put one by one into a roll of paper which was made of a sheet of filter paper (13.4^{c.m.} × 10.4^{c.m.}) for spinning cocoons, and thus any possible loss, which might otherwise happen, was prevented by this. On the 9th day after finishing to spin cocoons, some were taken out from the roll of paper and the excrements left in it were collected for analysis. The cocoons thus gathered were cut, and the pupae were taken out, some of which were dried up and analysed, and the rest were again put into another roll of filter paper to avoid any loss, which might happen when the moths appeared. On their appearing, they were collected and instantly killed by sulphuretted hydrogen gas, ground in a mortar into fine powder.

1. C. VANEY and F. MAIGNON: Compt. rend, 140, 1192-95.

2. E. ABDERHALDEN and WOLFGANG WEICHARDT: Zeitschr. f. physiol. Chemie 1909, 59 174 .

The larval and pupal skins cast off on metamorphosing were collected and analyzed.

B. Analytical part.

I. GENERAL ANALYSIS.

(1) Total nitrogen.

The total nitrogen of silkworms, pupae, moths, skins and excrements were determined with the following results :—

GRAMS IN 100 INDIVIDUALS.

	The results of the analysis conducted in 1909. (Koishimaru.)	The results of the analysis conducted in 1910. (not the same sample, but the variety is the same.)
The mature silkworms	4.79	4.76
Pupae	1.76	1.78
Moths	1.39	1.36
Cocoons	2.96	3.03
Larval skins cast away on pupating	0.07	0.19
Pupal skins cast on moths appearing	0.14	0.11
Urine of moths	0.26	0.48
Excrements of mature silkworms until be- coming pupae	—	0.26

From the results of the above experiment we may conclude that the silk-worm never lost its nitrogen in gaseous form while metamorphosing, as Dr. O. KELLNER and other authorities had confirmed ; because the sum of the total nitrogen in the pupae, the cocoons, and the larval skins cast on pupating, or the sum of the total nitrogen contained in the moths, the cocoons, the pupal skins and the urine of moths is nearly equal to the total nitrogen in the mature silkworms ; and the silkworms which belong to the same variety, also have nearly the same quantity of nitrogen.

(2) Albuminoid nitrogen.

To ensure obtaining results as exactly as possible, the fat contained in the sample was at first removed and then it was analyzed by Stutzer's method with the following results. :—

	Grams in 100 individuals.	Percentage in dry matter.
Silkworms	3.66	8.64
Pupae	1.21	6.03
Moths	0.996	7.68

The albuminoid nitrogen was greatly reduced while metamorphosing and the consumption must have been in great part due to the production of the cocoons.

(3) Nitrogen in phosphotungstic precipitate and its filtrate.

The filtrate of the copper hydrate precipitate was made to contain sulphuric acid as much as five per cent, and then phosphotungstic acid was added to precipitate basic nitrogen. The precipitate thus formed, was filtered after 24 hours standing and its nitrogen was determined by KJELDAHL'S method. The filtrate was evaporated to a small volume, decomposed by conc. H_2SO_4 and the nitrogen was also determined by Kjeldahl's method, with the following results:—

	Nitrogen in the phosphotungstic ppt.		Nitrogen in the filtrate of the phosphotungstic ppt.	
	Grams in 100 individuals.	Percents in dry matter	Grams in 100 individuals.	Percents in dry matter.
Silkworms	0.47	1.11	0.64	1.49
Pupae	0.17	0.85	0.38	1.89
Moths	0.13	0.96	0.24	1.84

(4) Nitrogen soluble in water.

4 grams of the sample, the fat of which had been removed, were warmed with 100^{c.c.} of water, filtered, well washed with water and filled up to 200^{c.c.} 50^{c.c.} of which were taken and the total nitrogen was determined with the following results:—

	Grams in 100 individuals.	Percent in dry matter.
Silkworms	1.32	3.12
Pupae	0.55	2.74
Moths	0.40	3.11

(5) Carbohydrate.

The sample was mixed with hydrochloric acid, which was prepared by diluting *HCl* (Sp. gr=1.125) so as to contain 20^{c.c.} of it in 200^{c.c.} of water, and boiled for two hours under a reverted cooler; then phosphotungstic acid was gradually added to remove organic bases which would otherwise prevent the obtaining of a red precipitate of cuprous oxide when FEHLING'S solution was added. The precipitate was removed after standing for 24 hours, and the filtrate was nearly neutralized with caustic soda. Then the carbohydrate was determined by the ordinary gravimetric method with the following results.

	Grams in 100 individuals.	Percent in dry matter.
Silkworms	2.11	4.98
Pupae	0.88	4.37
Moths	0.74	5.71

(6) Ether-extract (Fat).

Fat was determined by Soxhlet's method with the following results:—

	Grams in 100 individuals.	Percent in dry matter.
Silkworms	7.11	16.78
Pupae	8.72	43.45
Moths	3.14	24.21
Cocoons	0.07	0.42
Excrements	0.08	2.36

From the above results, we can say, the silkworm accumulated fat on changing into the pupa, and the quantity reached its maximum when changed into the pupa, but afterwards it was suddenly reduced to a small quantity, as E. CONOREUR confirmed.

II. SPECIAL ANALYSIS.

The sample was mixed with three times of conc. hydrochloric acid, boiled for 12 hours under a reverted cooler, then filtered from insoluble parts and diluted to 200^{c.c.}. The filtrate was analysed with the following results:—

The following figures are given in grams for 100 individuals.

	Silkworms.	Pupae.	Moths.	Cocoons.
Insoluble part	2.39	1.07	0.80	0.08
Soluble part	39.97	18.99	12.17	16.22
Total nitrogen	4.82	1.37	1.34	2.89
Nitrogen in the phosphotungstic acid ppt. ..	1.03*	0.33*	0.33*	0.18*
Nitrogen in the filtrate of the phosphotungstic acid ppt.	3.41	0.89	0.83	2.60
Ammonia nitrogen	0.38	0.15	0.17	0.11

* Ammonia-nitrogen is excluded.

In 100 parts of dry matter:—

	Silkworm.	Pupae.	Moths.	Cocoons.
Insoluble part	5.94	5.33	6.17	0.50
Soluble part	94.06	94.67	93.83	99.50
Total nitrogen	11.38	6.83	10.33	17.73
Nitrogen in the phosphotungstic ppt.	2.43	1.64	2.54	1.10
Nitrogen in the filtrate of the phosphotungstic ppt.	8.05	4.43	6.39	15.95
Ammonia nitrogen	0.897	0.75	1.31	0.80

The above shows, when the silkworm changed into the pupa the greater part of its nitrogen was transformed into the cocoon, and the nitrogen thus transformed existed chiefly in the form of monoamino acids. The fact is clearly seen below:—

	Silkworms.	Pupae.	Moths.	Cocoons.
Total nitrogen	100.00	100.00	100.00	100.00
Nitrogen in the phosphotungstic acid ppt. (Diamino-nitrogen)	21.37	24.09	24.62	6.23
Nitrogen in the filtrate of the phosphotungstic acid ppt. (Monoamino-nitrogen) ..	70.54	64.96	61.94	89.97
Ammonia nitrogen	7.88	10.95	13.71	3.81

The pupa and moth were nearly the same in their constitution, but the content of the amino acids in the latter was reduced, and that of ammonia was, on the contrary, increased. The fact is thought to be that the protein of the silkworm was first split into amino acids, which were then used up to maintain the energy of the cells as carbohydrate or fat does, and as the pupa was not supplied with nitrogen, its content had to be reduced. The amino acid nitrogen not used up for growth being split off from its combination in the form of ammonia, its content in the moth was to be increased.

C. Summary of the Results.

1. General analysis.

The following figures count as grams for 100 individuals.

	Silkworms.	Pupae.	Moths.	Cocoons.	Larval skins cast on pupation.	Pupal skins cast on moths appearing.	Excrements of silkworms.	Juice of excre- ments of silkworms.	Urine of Moths.
Fresh weight	237.50	102.50	53.14	—	—	—	—	—	—
Air dry weight	46.43	22.56	13.77	18.47	0.76	1.03	4.31	—	—
Dry matter	42.36	20.07	12.97	16.30	0.63	0.72	3.39	—	—
Water	195.14	82.43	40.17	2.17	0.13	0.32	0.92	—	—
Ash	2.44	0.92	0.63	—	—	—	0.87	—	—
Fat	7.11	8.72	3.14	0.07	—	—	0.08	—	—
Carbohydrate	2.11	0.88	0.74	—	—	—	—	—	—
Total nitrogen	4.76	1.78	1.36	3.03	0.19	0.11	0.26	0.17	0.49
Water-soluble nitrogen	1.32	0.55	0.40	—	—	—	—	—	—
Albuminoid nitrogen	3.66	1.21	0.996	—	—	—	—	—	—
Nitrogen in the phosphotungstic acid ppt.	0.47	0.17	0.13	—	—	—	—	—	—
Nitrogen in the filtrate of the phosphotungstic acid ppt. .. .	0.63	0.38	0.24	—	—	—	—	—	—

In 100 parts of dry matter :—

	Silkworms.	Pupae.	Moths.	Cocoons.
Ash	5.76	4.09	4.86	—
Fat	16.78	43.45	24.21	0.42
Carbohydrate	4.98	4.37	5.71	—
Total nitrogen	11.23	8.87	10.49	18.58
Water-soluble nitrogen	3.12	2.74	3.11	—
Albuminoid nitrogen	8.64	6.03	7.68	—
Nitrogen in the phosphotungstic acid ppt..	1.11	0.85	0.96	—
Nitrogen in the filtrate of the phospho- tungstic acid ppt.	1.49	1.89	1.84	—

In 100 parts of Nitrogen :—

	Silkworms.	Pupae.	Moths.
Total nitrogen	100.00	100.00	100.00
Albuminoid nitrogen	76.19	67.99	73.12
Nitrogen in the phosphotungstic acid ppt. ..	9.87	9.58	9.15
Nitrogen in the filtrate of the phospho- tungstic acid ppt.	13.24	21.38	17.55

2. Hydrolyzed part.

Grams for 100 individuals .—

	Silkworms.	Pupae.	Moths.	Cocoons.
Insoluble part	2.34	1.07	0.80	0.80
Soluble part	39.97	18.99	12.17	16.22
Total nitrogen	4.82	1.37	1.34	2.89
Nitrogen in the phosphotungstic ppt.	1.03	0.33	0.33	0.18
Nitrogen in the filtrate of the phospho- tungstic ppt.	3.41	0.89	0.83	2.60
Ammonia Nitrogen	0.38	0.15	0.17	0.11

In 100 parts of dry matter :—

	Silkworms.	Pupae.	Moths.	Cocoons.
Insoluble part	5.64	5.33	6.17	0.50
Soluble part	94.36	94.67	93.83	99.50
Total nitrogen	11.38	6.83	10.33	17.73
Nitrogen in the phosphotungstic ppt. ..	2.43	1.64	2.54	1.10
Nitrogen in the filtrate of the phospho- tungstic ppt.	8.05	4.43	6.39	15.95
Ammonia Nitrogen	0.90	0.75	1.31	0.80

In 100 parts of nitrogen :—

	Silkworms.	Pupae.	Moths.	Cocoons.
Total nitrogen	100.00	100.00	100.00	100.00
Nitrogen in the phosphotungstic ppt. ..	21.37	24.09	24.62	6.23
Nitrogen in the filtrate of the phospho- tungstic ppt.	70.54	64.96	61.94	89.97
Ammonia nitrogen	7.88	10.95	13.71	3.81

D. Investigation on proteolytic Enzyme in the Silkworm.

Concerning the enzymes existing in the silkworm, several have been reported, and we have also written about the occurrence of some desamidizing enzyme¹ in the mature silkworm, pupa and moth. As shown in the foregoing experiments, the silkworm undergoes a great change in its constitution while metamorphosing, and these changes were supposed to be due to the action of some powerful proteolytic enzymes which existed in its body. To confirm this, the following experiments have been carried out.

(1) The autolysis of the silkworm.²

Thirty ripe silkworms (*a*), weighing 56.18_g when living and 12.55_g in the dry state, were all crushed in a mortar, dried up and then analyzed. This

1). T. TAKENOUCHI and R. INOUE in The Journal of the College of Agriculture, Tokyo Imperial Univertsy, 1909, Vol. I. No. 1, 15.
2). The experiment was conducted in 1909.

served as control. Other thirty mature silkworms (*b*) were also well ground in a mortar, boiled for about one hour with some water and kept at room temperature for 12 days in an ERLÉNMEYER'S flask with a cotton stopper, and with enough toluole as an antiseptic. Occasionally stirred. Then the toluole was separated by decantation and the residue was gradually dried up. After complete desiccation at 105° C, the mass was pulverized and analyzed. The third thirty silkworms (*c*) were treated just as (*b*) but without boiling. After 12 days standing, dried up at 105° C, and analyzed.

The results of the analysis were as follows:—

	Control (<i>a</i>)		Boiled (<i>b</i>)		Unboiled (<i>c</i>)	
	Grams for 100 silkworms.	Percentage.	Grams for 100 silkworms.	Percentage.	Grams for 100 silkworms.	Percentage.
Total weight	187.26	100.00	197.7	100.00	195.101	100.00
Dry matter.. .. .	41.836	22.34	37.61	19.02	39.161	20.07
Water	145.424	77.66	160.09	80.98	155.94	79.93

	Grams for 100 silkworms	In 100 parts of dry matter.	Grams for 100 silkworms.	In 100 parts of dry matter.	Grams for 100 silkworms.	In 100 parts of dry matter.
Total nitrogen	4.78	11.42	4.81	12.99	4.72	12.04
Albuminoid nitrogen	4.22	10.08	4.14	11.37	4.29	11.57
N in the phosphotungstic acid ppt.	0.27	0.64	0.29	0.81	0.15	0.39
N in the filtrate of the phosphotungstic acid ppt.	0.29	0.69	0.35	0.81	0.25	0.63
Ammonia nitrogen	0	0	0	0	0.05	0.12
Chitin nitrogen.. .. .	0.67	1.69	0.78	2.08	0.64	1.63

In 100 parts of nitrogen.

	Control (a)	Boiled (b)	Unboiled (c)
Total Nitrogen	100.00	100.00	100.00
Albuminoid nitrogen	87.68	86.62	90.55
N in the phosphotungstic acid ppt. .. .	5.63	6.13	3.22
N in the filtrate of the phosphotungstic acid ppt. .. .	6.09	7.83	5.26
Ammonia nitrogen	0	0	1.02
Chitin nitrogen.. .. .	13.94	16.24	13.52

(2) The autolysis of the pupa.¹

Each thirty pupae, (a),² (b) and (c), were respectively treated just as in the foregoing case, but after one month's standing they were analyzed with the following results :—

	Control (a)		Boiled (b)		Unboiled (c)	
	Grams of 100 pupae.	Percentage.	Grams of 100 pupae.	Percentage.	Grams of 100 pupae.	Percentage.
Total weight	102.50	100.00	101.00	100.00	101.42	100.00
Dry matter.. .. .	20.07	19.58	18.21	18.03	16.37	16.14
Water	82.43	80.42	82.79	81.97	85.05	83.86
	Grams of 100 pupae.	In 100 parts of dry matter	Grams of 100 pupae.	In 100 parts of dry matter.	Grams of 100 pupae.	In 100 parts of dry matter.
Total nitrogen	1.78	8.87	1.77	9.74	1.64	10.01
Albuminoid nitrogen	1.21	6.03	1.25	6.87	1.25	7.61
Nitrogen in the phosphotungstic acid ppt. .. .	0.17	0.85	0.10	0.55	0.07	0.44
Nitrogen in the filtrate of the phosphotungstic acid ppt. .. .	0.38	1.89	0.42	2.31	0.32	1.95

1. The experiment was conducted in 1910.
2. The results of the general analysis of the pupae on page 8 must be referred to here, because the sample was the same

In 100 parts of nitrogen :—

	Control (a)	Boiled (b)	Unboiled (c)
Total nitrogen	100.00	100.00	100.00
Albuminoid nitrogen	67.94	76.21	76.22
Nitrogen in the phosphotungstic acid ppt. ..	9.55	5.65	4.27
Nitrogen in the filtrate of the phospho- tungstic acid ppt. }	21.35	24.18	19.51

On account of the sample being scanty ammonia and chitin nitrogen was not determined in the latter case.

In the above two cases nitrogen in the phosphotungstic precipitate and that in its filtrate in the unboiled parts were much reduced in quantity, and especially in the former case ammonia was produced. The fact shows that the protein in the unboiled silkworm and pupa was gradually split off into amino acids which were transformed into ammonia, and the action may be attributed to the presence of some proteolytic enzyme, but concerning the enzyme further observations are necessary.

E. Conclusions.

1. The chemical composition of the silkworm is greatly changed in producing the cocoon, while the difference between the pupa and moth is not so striking.
2. The silkworm never loses its nitrogen in gaseous form while metamorphosing.
3. Fat is accumulated when the silkworm pupates, but during the pupal and moth stage, the greater part of it is consumed.
4. The nitrogen in the filtrate of the phosphotungstic acid precipitate is more in quantity in every stage of the metamorphosis than the nitrogen in its precipitate, especially nitrogen in the cocoon consists chiefly of the former.
5. In the pupal and moth stage, the waste of body protein is repaired

with amino acids, and a part of the latter is further transformed into ammonia.

6. Splitting of the protein in the silkworm is caused by the action of some proteolytic enzyme.

In conclusion I heartily thank Mr. C. TSUJI, the expert of the Tokyo Sericultural Institute, for kindly giving me abundant quantities of silkworms reared by himself.

On the Preparation of Natto.

BY

S. Muramatsu.

There are several kinds of *natto* prepared in Japan, but here I mean common *natto*, which is a kind of vegetable cheese made by fermenting boiled soya beans wrapped in rice straw and set in a warm cellar for one or two days. Thus the product becomes white and mucilageous by the development of bacteria. *Natto* is consumed as a by-food after having been mixed with table salt and several stimulants, of which amongst others powdered mustard is preferred. It is chiefly consumed in Tokyo and the north-east districts of Japan, and for the production of it Aizu is the noted place. It is consumed in Tokyo in the summer time, but in the north-east districts during the winter time, as these are rather poor in vegetables at that season.

There exist several studies on *natto* so far, as to its constituents and the micro-organisms growing on it, but no exact investigation is known about its preparation. So, its manufacturers suffer under many difficulties in preparing *natto* of good quality; for this reason, I was induced to make a study of the method of preparing it and on several other points. Besides, I think it is very useful to prepare *natto* of good quality and increase its consumption by the people, as it is a very good and economical food stuff, being cheap and containing much protein; especially in our country, where rice is the principal food.

I. Soya Beans.

Soya beans are the principal raw material of *natto*. There are numerous varieties of soya beans cultivated in Japan, which, for instance, we can distinguish by their colour as yellowish white, green, black, spotted, etc. I

prepared *natto* with these different kinds and could not find a more suitable kind than the small yellowish white bean.

The beans which serve for the preparation of *natto* are first sorted, and all that are broken or imperfectly developed are picked out; besides, it is better to sift them through sieves with proper meshes to separate too small or too large ones. They are then washed and allowed to steep in clean water for several hours, after that they are boiled in a large iron kettle with sufficient water for *ca.* 5 hours. Thus the beans become moderately soft and their colour darker.

Their constitution was as follows :

In 100 pts. air-dry beans :—

Moisture	7.14
Dry matter	92.86

In 100 pts. dry matter :—

Crude protein	50,156
Crude fat	22,453
Crude fibre	6,420
N-free extract	11,871
{ Soluble in water Insoluble in water 4,329 7,542	
Ash	3,600
Total N	8,025
Albuminoid N	7,953
{ Soluble in water Insoluble in water trace. 7,953	
Non-albuminoid N	0,072

II. Rice Straw.

Rice straw is used for the wrapper of the boiled soya beans. Fresh straw is preferable to old, as its smell is better than that of the latter. The

straw is cleaned by taking the muddy leaf away from the under part of the stem and then washed with clean water; afterwards, it is well tied at its two ends, leaving several inches apart, and bundled after filling the bag with the beans. As to the reason of using straw for the preparation of *natto*, it was considered that the straw supplies the proper bacteria to the beans; but I do not think this the sole reason, for we can prepare it another way, as, for instance, by setting it in a sterilized Petri-dish or in a basket. When it is made in a basket, which after filling it with beans is put in a warm cellar covered with a straw mat, it is called *basket-natto*. From this and other facts it is reasonable to consider the principal objects of using straw for the preparation of *natto* to be:—

1. The supply of the good aroma of straw to *natto*.
2. To take away ammonia from *natto*.
3. To offer good ventilation of air to the loosely packed beans.

The bacteria which produce *natto* from soya beans are always present on the surface of the beans, and their spores being very hardy against high temperature, they are not easily killed by boiling, as we can see from the following experiment: The grains which were boiled for several hours are taken in sterilized Petri-dishes after each hour and placed in the incubator at 42°C . By this means, I found that the beans which were boiled for 8 hours become *natto* rich in mucilage and with good aroma. The fact that the *basket-natto*, which does not come in touch with straw, does not sell as well as common *natto*, tells us that its quality is inferior to the latter. The main reason is that its flavour is not so good as that of common *natto*, for, when we prepare it in the straw bundle its flavour is always superior to made in a Petri-dish, as it contains an aroma somewhat like that of straw. So, I think that the straw which is used as a bag for the beans gives its good aroma to *natto*.

When the bacteria grow on the beans they produce so much ammonia that we can perceive it by its peculiar smell. As the straw absorbs ammonia, the smell of it is more feeble when we use straw bundle than in the case of a glass dish. We can understand this fact when we see that the straw which has been used as a bag always contains much more ammonia than fresh material, and *natto* made in the dish is richer in it than that from the bundle.

	Amount of Ammonia.
In the fresh straw	0,035%
In the straw used as wrapper	0,065 „
In <i>natto</i> made in a glass dish	0,235 „
In <i>natto</i> made in straw bundle	0,188 „

For these reasons, *natto* prepared in straw bundles must have a better flavour than any other, by taking its flavour from straw and giving off the disagreeable smell of ammonia to straw.

The bacteria producing *natto* want much oxygen for their proper growth, as it is an obligate aerobe. So, when we prepare it by heaping up many bundles, the interior ones become inferior in quality and also, the interior beans of a large bundle become less viscous than the outer parts.

For this reason, it is recommendable to use small bundles for the preparation of superior *natto*.

III. Cellar.

The cellar for the preparation of *natto* is made with bricks or with wooden piles surrounded with thick layers of straw and the walls plastered with mud; the entrance is furnished with a thick door preventing the air to enter. Along the inside of the wall a long shelf two feet wide is set up at the height of about two feet and one or two large hearths are made on the floor for the purpose of warming the room.

IV. The Preparation of Natto.

For the preparation of *natto* the soya beans are sorted first and all beans that are broken or imperfectly developed are picked out.

After washing with clean water, they are soaked for several hours and boiled in an iron kettle until they become moderately soft. (*ca.* 5 hours.) The boiled beans are put into the straw bundle while they are still hot, and the bundles are placed, standing obliquely, on the shelf in the cellar, which is previously warmed by charcoal to about 40° C. The cellar is then shut up carefully, to avoid the circulation of air; thus, the beans become *natto* after one or two days and are ready for consumption.

V. The Microbes of Natto.

As to the micro-organisms of *natto* several authors have made investigations.

Dr. YABE isolated three species of micrococci which formed yellow, orange, and white colonies respectively, and a bacillus which is not motile, liquefying gelatine and producing a greenish fluorescence. He attributed the production of the characteristic aroma of *natto* to the development of the micrococcus which produces yellow colonies; but no explanation was given about the formation of the viscous substance.

Dr. SAWAMURA isolated various kinds of bacilli and micrococci from *natto* and regarded the following two bacilli as the chief microbes for the production of *natto*.

Bacillus No. 1 is a motile and facultative ærobe. *Natto* produced by this bacillus had a good taste and aroma, but its viscosity was not so great as that produced by the other. The author gave the name of *Bacillus natto* to this bacillus, considering it as the chief microbe in the fermentation.

Bacillus No. 2 is a rarely motile and facultative ærobe. *Natto* produced by this bacillus showed a stronger viscosity but a less nice taste and aroma than that produced by the *B. natto*; he recognised it as a variety of *Bac. mes. vulgatus*. Thus, he concluded that for the formation of good *natto* both bacilli must be present.

Mr. MÖNZEN isolated several kinds of bacteria, among them one bacillus to which Dr. OMORI gave the name of *Bacillus viscosus natto* and which, he said, is the principal microbe that produces strong viscosity. The two kinds of bacilli which he named *Bacillus odorans natto 1.* and *Bacillus odorans natto 2.*, produce good aroma in *natto*; and another one which he named *Pseudomonas odorans natto*, produces also good aroma. The latter three did not produce good *natto*, unless the material is inoculated also with *B. viscosus natto*. Thus the author concluded that there are necessary for the preparation of *natto* at least two kinds of bacteria, one producing the peculiar aroma and the other strong viscosity.

Mr. MUTO isolated several bacteria and concludes that only one bacillus belonging to the *B. subtilis* group is necessary for the production of *natto*.

I investigated also several kinds of *natto*, prepared in Tokyo, Aizu, and Morioka, and found that they all contain the same micro-organisms, amongst which the following three bacilli are the principal ones. Several other bacilli are not suitable for the preparation of *natto*, as they produce bad colour or smell, and make the *natto* unfit for eating.

Two micrococci were found, one of which was analogous to *Mic. flavus*, and the other producing a translucent colony on agar plate-culture : but, both these micrococci having no relation to the preparation of *natto*, I gave up their further investigation.

BACILLUS No. 1.

This bacillus develops most energetically at high temperature (40-50° C.) and produces the best quality of *natto*, providing much mucilage and good aroma.

Form :

The cells grown in bouillon at 40° C. are 1 μ thick and 5-8 μ long.

Motility :

It moves energetically, providing long cilia around its body.

Spore :

An oval spore is formed principally in one end of the cell, which is 0.8 μ thick and 1.6 μ long ; the formation of the spore requires 4 hours at 42° C. and germination of it begins equatorial after 2½ hours at the same temperature.

Oxygen :

Obligate ærobe.

Colouring :

It is coloured readily with aniline colours and also after GRAM'S method.

Bouillon culture :

Bouillon remains almost clear after its development, and a strongly folded film, coloured slightly grayish brown, is formed after 10 hours at 38° C.

Sugar bouillon becomes slightly turbid changing its reaction to acidic at the beginning, which turns alkaline gradually ; gas is not formed.

Peptone-water culture :

It produces a grayish white film on its surface and the liquid becomes slightly turbid.

Gelatine plate-culture :

Small white colonies are formed which liquefy it quickly.

Gelatine stab-culture :

It develops vigorously at the surface and liquefies gelatine in the shape of a funnel ; the liquefied part remains transparent and a film is formed.

Agar plate-culture :

White and mealy-looking colony, that has a rough wristle at its centre but delicate at its edge, spreading very rapidly at 40° C.

Agar slope-culture :

Colony develops along the line and spreads rapidly all over the surface with mealy appearance ; the condensed water remains transparent with a film on its surface, but no sediment.

Potato culture :

Elevated colony is formed in the beginning, which spreads soon over the whole surface of the medium ; the colour of the colony is yellowish brown and it is folded with mealy appearance, the medium becoming brown.

Milk culture :

It is coagulated at first and is dissolved again.

H₂S :

Is formed.

Indol reaction :

Is not obtained from old bouillon culture.

Reducing property :

It reduces methylene blue in bouillon but does not develop in the glucose nitrate medium.

Ammonia :

Is formed in the culture of bouillon and soya beans.

Enzyme :

Diastase and proteolytic enzyme of tryptic nature are recognised.

Behaviour to temperature :

It develops very vigorously at $50^{\circ}C.$, but not at $60^{\circ}C.$ It is killed at $60^{\circ}C.$ after two hours, and after one hour at $80^{\circ}C.$

The resistance of the spores against heat is very strong, for it takes one hour to kill them in KOCH's steam-steriliser.

Behaviour to several compounds :

Table salt :

In bouillon containing 15% *NaCl* it develops slowly, but not in 20% solution.

Alcohol :

It develops in bouillon containing 4%, but not in 5% alcohol.

The spore is not killed readily with alcohol, as it is still alive after ten days and more, when put either in 50% or absolute alcohol at $20^{\circ}C.$

HCl :

It develops in bouillon containing 0.025% *HCl*, but not in 0.05%. The spore which is put in 3% *HCl* is alive after one day, but not after two days. In 4% *HCl* it is alive after one hour, but not after two hours.

Acetic acid :

It develops in bouillon with same concentration as hydrochloric acid.

The spore is not killed by glacial acetic acid after 10 days and more.

NaOH :

It develops in bouillon containing 0.2% *NaOH*, but not in 0.3%.

The spore is killed when it is put in 35% solution after one day.

Phenol :

It develops in bouillon containing 0.1% phenol, but not in 0.2%.

The spore is not killed after ten days when put in 5% solution.

Corrosive sublimate :

It develops in bouillon containing 0.0025% *HgCl₂*, but not in 0.005%.

The spore is killed after 50 minutes when it is put in 0.1% solution, but it was alive after 40 minutes in the same solution.

This bacillus may be the same as those which Dr. SAWAMURA represented as *Bacillus* No. 2 and *Bacillus viscosus* Ōmori, and also that which Mr. Muto

thought was the only bacterium which produces *natto*, though there are several differences in its behaviour investigated by these authors.

BACILLUS No. 2.

This bacillus develops most energetically at high temperature and produces *natto* of the best quality, forming much mucilage and a rather higher aroma than *Bacillus* No. 1.

Form :

The cells grown in bouillon at $40^{\circ}C$. are $0.8-1\mu$ thick and $4-10\mu$ long.

Motility :

It moves vigorously providing long cilia around its body.

Spore :

An oval spore is formed in one end of the cell, and it is 0.8μ thick and 2μ long.

The spore wants 4 hours at $42^{\circ}C$ for its formation and it germinates equatorial after $2\frac{1}{2}$ hours at the same temperature.

Oxygen :

Obligate ærobe.

Colouring :

The cell is coloured easily by aniline colouring matters and also after GRAM'S method.

Bouillon culture :

Bouillon remains almost clear after its development, and a strongly folded film of slightly grayish brown is formed after twelve hours at $38^{\circ}C$.

Sugar bouillon becomes slightly turbid, changing acidic at the beginning, which turns alkaline gradually ; gas is not formed.

Peptone-water culture :

It produces a grayish white film on its surface and the liquid becomes slightly turbid.

Gelatine plate-culture :

Small white colonies are formed which liquefy it quickly.

Gelatine stab-culture :

It develops quickly on the surface and liquefies gelatine in the shape of a funnel; the liquefied part remains transparent and a film is formed.

Agar plate-culture :

There is formed a white and mealy-looking colony with rough wristle at its centre but delicate at its edge, spreads very quickly at 40° C.

Agar streak-culture :

Colony develops along the line and spreads rapidly all over the surface with mealy appearance.

The condensed water remains transparent with a folded film on its surface but no sediment.

Potato culture :

Elevated colony is formed in the beginning, which soon spreads over the whole surface of the medium; the colony is folded and has brownish yellow colour and mealy appearance; the medium becomes brownish gray.

Milk culture :

It is coagulated at the beginning and is dissolved again.

H_2S :

Is not formed.

Indol reaction :

Is not obtained from old bouillon culture.

Reducing property :

It reduces methylene blue in bouillon and produces ammonia by reduction of nitric acid in the glucose nitrate medium.

Ammonia :

It is formed in the culture of bouillon and soya beans.

Enzyme :

Diatase and proteolytic enzyme of tryptic nature are recognised.

Concerning the behaviour against heat and several compounds as formerly mentioned, there is not much difference with *Bacillus* No. 1.

This bacillus may be the same as that which Dr. SAWAMURA named *Bacillus natto*, though there are several differences in its behaviour. As this bacillus does not produce any mucilage at low temperature (say 35° C) he

thought it, perhaps, to be one which produces aroma peculiar to *natto*; but, as I mentioned already, this bacillus produces much mucilage at higher temperature and makes good *natto* with high aroma.

BACILLUS No. 3.

This bacillus develops most energetically at $40^{\circ}C$, and when it is developed on boiled soya beans at this temperature, it produces good *natto* with strong viscosity and good aroma; but its mucilage is somewhat less than Bacillus No. 1 and Bacillus No. 2.

Form :

The cells grown in bouillon at $40^{\circ}C$. are 1.2μ thick and $6-10\mu$ thick

Motility :

It moves providing long cilia around its body.

Spore :

An oval spore is formed in one end of the cell, which is 1μ thick and 1.5μ long.

The spore is formed after 4 hours at $42^{\circ}C$. and germinates equatorial after $2\frac{1}{2}$ hours at the same temperature.

Oxygen :

Obligate ærobe.

Colouring :

It is coloured readily with aniline colours and also after GRAM'S method.

Bouillon culture :

Bouillon becomes slightly turbid, a brittle film of slightly grayish brown colour is formed after ten hours at $38^{\circ}C$. and produces a small amount of sediment. The film is broken easily by shaking and sinks to the bottom.

Sugar bouillon changes to slightly acidic at the beginning and turns slightly alkaline afterwards.

Peptone-water culture :

It becomes slightly turbid and forms a yellowish white film on its surface. Gas is not formed.

Gelatine plate-culture :

Small white colonies are formed which liquefy it quickly.

Gelatine stab culture :

It develops on the surface at the beginning and liquefies gelatine in the shape of a funnel, afterwards thoroughly.

Agar plate-culture :

White colony with rough wristle at its centre but delicate at its edge, spreads very rapidly at 40° C.

Agar slope-culture :

The colony develops along the line and spreads rapidly in the shape of a feather; the condensed water is transparent with a film on its surface, but no sediment.

Potato culture :

A yellowish gray colony is formed, somewhat elevated in the beginning; it spreads soon over the whole surface of the medium. The colony has strong viscosity and it is folded shallower than *Bacillus No. 1* and *Bacillus No. 2*, the medium becoming gray.

Milk culture :

It is coagulated and dissolved again.

 H_2S :

Is produced.

Indol reaction :

Is not obtained from old bouillon culture.

Reducing property :

It reduces methylene blue in bouillon, and ammonia is formed by the reduction of nitric acid in the glucose nitrate medium.

Ammonia :

Is formed in the culture of bouillon and soya beans.

Enzyme :

Diastase and proteolytic enzyme of tryptic nature are recognised.

The behaviour to heat and several compounds is almost the same as with *Bacillus No. 1*, although there are some differences.

This may be the same bacillus as *Bacillus grossus*, but as there is no detailed description of it, I cannot make a precise comparison.

VI. The Application of cultured Bacteria for the Preparation of Natto.

As mentioned already, when we prepare *natto* in a glass dish at *ca.* 38°C. inoculated with *Bacillus No. 1* it has some viscosity, while others have not, but the aroma was inferior to that made in straw bundles, for it does not touch with straw. At 45°C. all bacilli produce *natto* of fine quality, providing strong viscosity and good aroma; the aroma produced by *Bacillus No. 1* was the best, while *Bacillus No. 2* produces a rather strong smell of ammonia, and that of *Bacillus No. 3* was the worst. Moreover, I prepared *natto* according to the common way differing only on the point of inoculating these bacilli separately and also mixing them with one another. The result was that *natto* which was produced by the inoculation of *Bacillus No. 1* was the best, as it has much mucilage and fine aroma, while *Bacillus No. 2* produced an inferior and *Bacillus No. 3* the worst quality. *Natto* produced by the inoculation of mixed bacilli was not so good as that produced by each bacillus; so, there is no necessity that two or more bacilli be present for the formation of good *natto*. By the inoculation of cultured bacteria we can entirely avoid failures and can prepare good *natto* by selecting the bacteria. Otherwise, it is sufficient to put it in the cellar for only one day, after which the *natto* will be ready for consumption. I recommend, therefore, the use of the pure culture of proper bacteria in the following way:

The bacteria developed on the slope culture medium of agar are mixed with juice produced by the boiling of beans. This is poured over the surface of boiled beans while they are still in the kettle, the further processes being the same as usual. There is no necessity of mixing several bacilli.

VII. Natto as a By-food.

As *natto* is prepared from soya beans which are rich in protein and carbohydrates, it contains yet much protein and carbohydrates; the nutritive value of it is greater than that of boiled soya beans, for it is rich in soluble matters produced by the micro-organisms.

The composition of *natto* differs exceedingly with age, but its mean composition is as follows: (Compare with the composition of boiled soya beans.)

In 100 pts. of fresh *natto*:

Moisture	53,480
Dry matter	46,520

In 100 pts. of dry matter:

Crude protein	46,088
Crude fat	20,216
Crude fibre	6,140
N-free extract	3,348
{ Soluble in water 2,495	
{ Insoluble in water 0,853	
Ash	5,010
Total N	7,374
Albuminoid N	5,458
{ Soluble in water 1,141	
{ Insoluble in water 4,317	
Non-albuminoid N	1,916

The micro-organisms which grow on the soya beans secrete trypsin and diastase; so, when we take it together with several foods rich in protein or starch, they may be digested more rapidly than when they are taken alone.

I express many thanks to Dr. SATO, Director of our College¹, who helped me in determining the quality of *natto* that I prepared, and also to Mr. N. NITTA and Mr. Y. TANAKA, who assisted me in these investigations.

1. College of Agriculture and Dendrology, Morioka.

On the Chemical Composition of Saké.

BY

Teizō Takahashi and Gorō Abé.

Various substances have hitherto been reported as occurring in saké, but so far some important components have been entirely overlooked. Some authors write on the composition which is naturally derived from the fermentation of the mash, others, on the other hand, on the constituents which have been derived from the vat or the cask used for storage or transportation of saké. These vats and the casks are made from wood of *Cryptomeria japonica* and therefore certain components of the material of the vessels must be dissolved and present in the beverage. CH. KIMOTO¹ reported on sughi-oil in 1903, and N. NAGAI² and T. KIMURA made some researches on its chemical composition, and found protocatechin, a chinon like substance, vanillin, and a kind of terpene, and they have affirmed the occurrence of the same substances in the beverage. The terpene, mentioned by the above authors, was proved by K. KEIMATSU³ to be a sesterpene, and he added as a new component of *Cryptomeria japonica* a phenol-like substance, having reducing property. In the same year YAMAMOTO and ISHIKAWA⁴ made studies on the same reducing substance as regards its influence in determining the reducing sugar in the beverage.

On the proper composition of saké K. KEIMATSU⁵ reports on the occurrence of furfurol in saké and one of us⁶ made research on the same

1. Bulletin of the College of Agric. Tokyo Imp. Univ. Vol. 4. p. 403.
2. In a speech made at the Tokyo Chemical Society (1904).
3. Yakugakuzasshi (Journal of the Pharmaceutical Society of Japan). March, 1905.
4. do: Sep. 1905.
5. do: Dec. 1904.
6. TEIZŌ TAKAHASHI: The Journal of the Agric. Society of Japan. May, 1905.

substance and found that in young saké, or shortly after the pressing of the mash, there was no furfural, or if present, it was but a trace, while in aged saké, i.e. after the storage during summer, it was always a constant component of it. H. NISHIZAKI's¹ determination however, of furfural, in the same year, arrived at somewhat different results from the writers. On the other hand, H. Itō's experiments recently made on this subject² with 111 samples of the beverage affirmed the conclusion of the writers. A few years ago, one of us³ made a report in respect to the fusel oil in the beverage and pointed out that the quantity of the substance must be reckoned on as a factor for judging the quality of the beverage. Moreover, a small quantity of methylalcohol was proved to be present in ordinary saké,⁴ and a somewhat evident quantity of *methyllactate* in certain samples of saké was found by one of us.⁵

In the report of H. NISHIZAKI's on free acids⁶ and sugars⁷ of saké he mentions that the latter consisted chiefly of glucose, while K. SUDA,⁸ already sixteen years ago, made experiments on the same subject and asserted that the sugar consists chiefly of maltose. It seems to the writers that both authors may be right because they used only a limited number of samples and therefore arrived at altogether opposite results. If they had examined a wider range of samples they would probably have found out that their results were one-sided. K. KEIMATSU⁹ and SHIMIZU made a speech at the meeting of the Pharmaceutical Society of Japan on the presence of aldehyd, fusel oil, succinic acid, lactic acid and acetic acid in saké.

On amino-acids, however, no one has reported as yet and therefore we present here the results of our investigations about amino-acids and other components. The general chemical composition of the sample was as follows:

1. Jour. of the Pharmac. Soc. of Japan. Nov. 1905.
2. Jour. of Tokyo Chemical Society, Vol. 32, No. 7.
3. TEIZŌ TAKAHASHI: The Journal of the Agric. Soc. of the Japan. Apr. 1905.
4. TEIZŌ TAKAHASHI: Bull. of the Agric. Coll. Tokyo. Imp. Univ. Vol. 6, No. 4.
5. do. Vol. 7, No. 4.
6. Journal of the Pharmac. Society of Japan. May 1905.
7. do. May 1906.
8. do. Apr. 1890.
9. do. Dec. 1905.

1)	Total-N.	0.1865%.
2)	Protein-N.	
	a) STUTZER'S method.	0.0072%.
	b) RÜMLER'S method. ¹	0.00672%.
	c) Precipitate by Pb-acetate and Pb-oxide.	0.00447%.
	(BUNGENER u. FRIES. Zeit. f. d. ges. Brau. 1894. 69.)	
	d) Precipitate by basic lead acetate.	0.00435%.
3)	Non-albuminoid-N.	
	a) Ammonia-N. (WURSTER'S method).	0.00629%.
	b) Organic bases-N.	0.0598%.
	c) Other-N. (chiefly amino-acids).	0.1131%.
4)	Esters (as acetic ester).	0.0457%.
5)	Total acid (as succinic acid).	0.2666%.
	a) Non-vol. acid (as succinic acid).	0.2596%.
	b) Volatile acid (difference).	
	(as acetic acid).	0.00715%.
	c) Volatile acid (determined in the distillate).	
	(as acetic acid).	0.0216%.

According to the general acceptance, STUTZER'S and RÜMLER'S method will precipitate, in a certain degree, a part of the albumoses and peptones, beside proper protein, and therefore in the above table these two determinations give rather a higher percentage than that of basic lead acetate, or that precipitate formed by lead acetate and lead oxide. For this reason we can assume for a certainty the presence of albumoses and peptones in saké, still further we can affirm directly the presence of both substances. If we take 100–200 c.c of the beverage and after removing the protein by basic lead acetate and from this filtrate after removing lead by H_2S and evaporating to a small volume, adding a sufficient quantity of Zn-sulphate to saturate the solution, acidifying with sulphuric acid, there will be formed a precipitate of *albumoses*. From the filtrate of albumoses, after removing zinc by H_2S and condensing to a small volume, this solution will contain *peptones*, which can be proved easily by biuret reaction.

1. RÜMLER: Deutsch. Zeit. Ind. 1898. 1729. The difference between the contents of nitrogen gained by STUTZER'S method and that of the precipitate made by lead acetate is shown from the above table to be 0.0027%, and this must be the least quantity of the nitrogen of albumoses and peptones.

1) Organic bases.

For the isolation of the bases 10 liters of the sample were evaporated under reduced pressure, and at $60^{\circ} C$ to a small volume, almost equal to $1/5$ of the original. After this operation the protein substance was removed by basic lead acetate, and researches were made about the bases according to KOSSEL's method, but of histidine only a trace was found, and although the characteristic crystals of the chloride were not obtained, PAULY's diazo reaction (1904) was ascertained. Picrate of lysine, about 1.1 gramm, was obtained as fine needles and platy crystals, which melted at $230^{\circ} C$ (uncorr.), indicating that it contains still some impurities. Arginine was not found.

2) Mono-amino-acids.

Ten litres of saké were subjected to the esterifying process after FISCHER's well known method to isolate mono-amino-acids.

a) Under pressure below 20. m.m.

(Esters prepared from 10 litres saké).

First fraction, $60-92^{\circ} C$.	1.0 gm.
Second „ $92-150^{\circ} C$.	1.5 „
Third „ $150-235^{\circ} C$.	Trace.

b) Under pressure below 18. m.m.

(Ester prepared from 10 liters of saké).

First fraction, below $40^{\circ} C$.	0.6 gm.
Second „ $40-60^{\circ} C$.	0.00 „
Third „ $60-98^{\circ} C$.	3.0 „
Fourth „ $98-150^{\circ} C$.	4.5 „

From the first fraction of the series (a), 0.2 grms of alanine, 0.2 grms of leucine, and 0.1 gm of proline were obtained. In the second series 0.2 grms of alanine from the first fraction and 1 g. of leucine, and a trace of proline from the third fraction, were obtained.

The alanine obtained from the first series was added to the same from the second series, and after purification analyses were carried out. It was very sweet, giving a melting point of $243-245^{\circ} C$ (uncorr.) and decomposed at the point with evolution of gas. The result of analysis was as follows:

Substance taken	0.172.
Nitrogen = 12 c.c. (at $15^{\circ} C$. 760 m.m.)	

Calculated as $C_3 H_7 N O_2$ $N=15.67\%$.

Found $N=14.51\%$.

Proline was bitter in taste and its copper salts contained two components, one of which dissolved in absolute alcohol, while the other does not, indicating the presence of active and inactive proline.

Leucine¹ was slightly bitter, so that it was washed repeatedly with absolute alcohol. It melted at $290-293^\circ C.$ (uncorr.), changing into brown at $270^\circ C.$ already. It decomposed at the melting point with emission of gas. The analytical result was as follows:

Substance taken. 0.1226 gm.

Nitrogen $10.7 C. C.$ (at $11^\circ C.$ 758 m.m.).

Substance taken. 0.1102 gm.

$C O_2$ 0.2165 „

Calculated as $C_6 H_{13} N O_2$ Found.

$N=10.85$ 10.46

$C=55.80$ 53.57

$H=10.07$ 9.73

Phenylalanine and glutamic acid were not found, but a trace of aspartic acid was probably present.

Leucinimide ($C_{12} H_2 N_2 O_2$). The residue remaining after the removal of all esters was treated with acetic ester, which dissolved a part of it. When the dissolved part was evaporated, there remained hexagonal and quadratic plates, which tasted very bitter. The platy crystals were dissolved again in acetic ester and after evaporation, ether and ethylalcohol were added, but no crystal was found. So we could not assert the presence of leucinimide.

Tyrosine. The presence of this substance was easily proved by MILLON'S reaction with saké after the removal of protein-substance. But for the isolation we followed BROWN² and WILLER'S operation, which they employed for the isolation of this substance from malt. Thus, the yield from three litres of saké amounted to about 0.1 gram. While as in other cases, ten liters of

1. The preparation of leucine perhaps was not pure, inasmuch as the above analytical data does not coincide with that of the calculated.

2. BROWN u. WILLER. Woch. für Brau. 1907. Nr. 11. S. 139.

the sample were evaporated to almost one fifth of the original volume of saké and left standing over-night in a cool place, there appeared the characteristic silky crystals of tyrosine amounting almost to six grams.

The tyrosine isolated gave strong red coloration by MILLON'S reagent, faintly red by PAULY'S¹ diazo reaction, distinctly red by WURSTER'S reaction² and DENIGE'S test.³ The analytical result was:—

Substance taken: 0.1048 gram.

Nitrogen. 6.8 c.c. (at 10° C. 762 m.m.).

Calculated as $C_9 H_{11} N O_3$ N.=7.77%.

Found. N.=7.79%.

Cystine. A small quantity of this substance was proved to be present in the filtrate obtained after removing the protëin substance of saké, but the quantity was too small to isolate it.

Tryptophane ($C_{11} H_{12} N_2 O_2$). This substance was obtained from 110 c.c. of saké by HOPKINS' and COLE'S⁴ method. The crystals were platy and bright, giving red coloration with bromine water and precipitated by phosphotungstic acid.

A few crystals were mixed with caustic potash and fusing subjected to dry distillation, pyrrol reaction was observed in this distillate. However, the presence of tryptophane in saké is limited only to young samples, or unaged saké. On this fact one of us has already reported⁵ in detail. On the cause of the disappearance of this substance in the aging process of saké, H. ITO⁶ has made some observations and arrived at the conclusion that the tryptophane in young saké is assimilated or rather decomposed by so-called AGING YEAST,⁷ *Willia anomala* varieties.

1. HOPPE-SEYLER'S Zeit. für ph. Chem. 42. 517 (1904).

2. Beautiful red color by acetic acid and natrium nitrite. (Ch. f. Ph. 1. 193. 1888).

3. Wine red by formaldehyd and $H_2 S O_4$. (Comptes rendus 130. 583. 1900).

4. Jour. of Physiol. 27. 418 (1902). 29. 451 (1903).

5. TEIZŌ TAKAHASHI. Journal of the Tokyo Chemical Society of Japan. Vol. 32. No. 3. 1911. Also this Journal.

6. H. ITO. Journ. of Tokyo Chemical Society of Japan. Vol. 32. No. 7. 1911. Also this Journal.

7. TEIZŌ TAKAHASHI. Journal of the College of Agric. Tokyo Imp. Univ. Vol. 1. No. 3.

Substances which dissolve in ether.**(1) *Succinic acid.***

Ten litres of saké were evaporated to about 400 c.c. under reduced pressure below $70^{\circ} C$. The syrup thus obtained was extracted with ether using KUMAKAWA and SUDO's extraction apparatus. From ether extract, ether was evaporated and dried for a long time in a desiccator containing sulphuric acid, there then appeared long platy crystals or monoclinic prisms in brown colored magma. The crystals were separated by filtration, and after washing very quickly with a small quantity of cold absolute alcohol, there remained almost colorless crystals, which melted at $181\text{--}182.5^{\circ} C$. (uncorr.). The substance acts very strongly acidic to litmus, has the characteristic taste of succinic acid and gives pyrrol reaction when subjected to NEUBERG's proof (1900–1901). The pure substance obtained from ten litres of saké was about 3 grams. From another 18 litres, 4.7 grams of the substance were obtained as raw product, which was brown in color, so that it was first neutralized with a 5% solution of sodium hydroxide, and after evaporating to the syrup absolute alcohol was poured on, there appeared almost spontaneously fine crystals of sodium succinate. The sodium salt thus obtained was washed repeatedly with absolute alcohol. It was silky white and gave a brown precipitate with ferric chloride.

(2) *Lactic acid*

The syrup obtained after the removal of the crystals of succinic acid was neutralized with zinc carbonate, becoming a thick pasty mass from which, after standing overnight, the separated crystals of zinc lactate were washed with absolute alcohol. The salt thus obtained was treated with sulphurated hydrogen to separate zinc, and the clear colorless solution of the acid gave intense reaction of UEFFELMANN's proof. Pure lactate of zinc was prepared from this pure acid, and after drying well in the desiccator the water of crystallization was determined, drying several hours at $105\text{--}110^{\circ} C$. The water of crystallization¹ amounted to 17.0%, almost equal to that of the inactive form of lactic acid.

1. The zinc salt inactive form containing 18.17% of water of crystallization (HOPPE-SEYLER's *Chemische Analyse*. 1909. S. 77.)

(3) *Tyrosol.* $O\ H.\ C_6H_4\ C\ H_2\ C\ H_2.\ O\ H.$

For the isolation of tyrosol we followed F. EHRLICH's¹ method. Ten litres of the sample were evaporated to almost 400 c.c. and after basifying with sodium bicarbonate, ether extract was made by SUDO and KUMAKAWA's extraction apparatus. The yield was 4 grams as raw product. It was dissolved again in absolute alcohol and decolorized with animal black. It behaved very strongly to MILLON's reagent and diazobenzolsulfonic acid, and faintly to ferric chloride² and DENIGE'S-MOENER's proof. Tyrosoldibenzoate prepared by the addition of benzoylchlorid and natronlye, melted at $113^{\circ}\ C.$ (uncorr.).

Summary.

The above statements are summarized here as below. The yield of the isolated substances from 10 litres of saké:—

- | | |
|---|------------------------|
| 1) Glycocoll. | Not found. |
| 2) Alanine. | 0.2 grams. |
| 3) Leucine | 0.6 „ |
| 4) Proline (active and racemic)..... | 0.1 „ |
| 5) Phenylalanine. | Not found. |
| 6) Glutamic acid. | „ „ |
| 7) Aspartic acid. | (Trace?). |
| 8) Leucinimide. | Not found. |
| 9) Tyrosine | 6.0 grams. |
| 10) Cystine. | Trace. |
| 11) Tryptophane (only occurs in young saké.) .. | 1.0 grm., or above. |
| 12) Lysine. | 0.25 grams. |
| 13) Histidine. | Trace. |
| 14) Arginine. | Not found. |
| 15) Tyrosol. ... | 4 grams (raw product). |
| 16) Succinic acid. | 3 „ (at the least). |

1. Ber. d. Deut. Chem. Ges. XLV. Heft 1, S. 139-146 u. Biochem. Zeit. 36. Band, 15. Heft, S. 477. 1911.

2. F. EHRLICH. Ber. d. Deut. Chem. Ges. 1911. XLV. Heft 1, S. 143.

- 17) Lactic acid (inactive) 3 grams („ „).
18) Albumoses and peptones.¹ Present.

The scarcity of the yield of amino-acids in comparison to the nitrogen contents of amino-acids obtained from calculation, must be ascribed to the insufficient vacuum, under which we have made fractions, namely rather high pressure (18-20 m.m.) but besides this, saké contains a rather high percentage of carbohydrate, which makes it difficult to isolate the esters.

In conclusion the writers must offer many thanks to their assistant MAKOTO SATŌ, for his faithful help during this research.

1. On the quantity of these two substances refer to the article "The property of the head etc" by TEIZŌ TAKAHASHI in this Journal.

On the Occurrence and Disappearance of Tryptophane in Saké.

BY

Teizō Takahashi.

In the article "On the chemical composition of saké," the writer¹ reported with G. ABÉ on the occurrence of tryptophane in saké as a component of it. Recently, H. ITÔ² affirmed that the main cause of the presence of this compound in saké is the result of the enzymic action of "koji" fungus, namely, *Aspergillus Oryzae*. During the "moto"-mash stage of saké brewing the enzymic action of "koji" fungus proceeds gradually, accompanied by bacilli forming lactic acid especially in the beginning of the process; therefore, the occurrence of this substance in "moto"-mash must be traced through several stages in the process. The presence of this substance was proved at several stages, using bromine water as shown in the table below:—

Stages.	Reaction of tryptophane.
Wakitšuki. (Starting).	Not found.
Kaniawa. (Very viscid bubbles).	„ „
Takaawa. (high bubbles).	„ „
Motówaké. (Separation of the "moto" mash). . . .	Evident.
Jukusei. (Finished "Moto").	Very strong.

In the mash of the principal or "moromi" process, this substance began to appear 10 or 13 days after the "tomé" operation, or end, and rather strong in the finished "moromi" mash and in young saké. After

1. This Journal. Vol. V. No. 2. p. 95; also Report of the Saké brewing Institute at Ōji, 1909. No. 18.
2. This Journal. Vol. V. No. 2.

this observation bromine reaction¹ was tried in about 149 samples of saké, of which 47 samples were old or aged saké, others were young. The existence of the substance was proved with certainty solely in the case of new samples. Recently the writer examined about 1600 samples of young saké and arrived at the same result as above, proving conclusively the presence of tryptophane in them.

The conclusion is that young or new samples from all localities of Japan contains tryptophane without exception; while in *old saké* i.e. *after the storage during summer time, it disappears*² or at least *we cannot find it directly in the sample*.

1. About 10 c.c. of Saké was taken in a test tube and saturated bromine water was added drop by drop shaking vigorously. The red color formed by tryptophane was absorbed by amylalcohol.

2. H. Irō has found the cause of the disappearance of this substance, and we believe with him, that the rôle played by so-called aging yeast or *Willia anomala* in saké is very important to cause this change in it.

Is there any Relation between the Property of the “Head” of the Saké-mash and the Quality of Saké.

BY

Teizō Takahashi.

In the industry of saké brewing we are told that the property of the “Head” of the fermenting mash has a great influence upon the quality of saké. Practically, when milky and thick bubbles appear on the surface of the fermenting mash, they are regarded as a symptom of good result. Recently, two mashes were fermented, applying two quite different varieties of saké yeast, *Sacch. saké* B. No. 34 and A No. 21, at the saké-brewing institute at Ōji near Tokyo. The original “Moto”-mashes, which were used for the isolation of these varieties of saké-yeast, were collected from different localities and amounted to 70 in number. After the comparative examination of their physiological properties, especially their fermentation products, B. No. 34 was adjudged the best yeast and A No. 21 the inferior kind.

The appearance of the fermenting mashes was quite different in both cases. i. e. in the vat, which contained the mash fermented by the yeast B No. 34; the rising of the temperature of the mash was very gradual after the “tomé”-stage; while before this stage the temperature rose rapidly and the fermentation process was very energetic and vice versa in the case of the yeast A No. 21. The quality of saké obtained from these mashes was quite different, and the one which was prepared by the variety B. No. 34 was the best of all preparations during this year. The analyses gave the following data:

Varieties of Yeast.....	B. No. 34.	A. No. 21.	Difference.
Quality of Saké.....	Superior.	Inferior.	

Amino-acids ¹	0.2488%.	0.2793%.	0.0305.
Total-N.	0.1081%.	0.1088%.	0.0007.
Protein-N.			
STUTZER'S method ..	0.0515%.	0.0490%.	0.0025.
RÜMPLER'S method..	0.0256%.	0.0248%.	0.0008.
Basic Pb-acetate ppt.	0.00915.	0.01001.	0.00086.
Dextrine.	0.5873%.	0.5851%.	0.0022.

The numerical difference of the protein nitrogen between the amount obtained from basic lead acetate precipitate and STUTZER'S or RÜMPLER'S method is worthy of remark. According to the general acceptance, in the determination of the protein matters by STUTZER'S or RÜMPLER'S method, beside proper protein matters certainly a part of albumoses and peptone precipitate accompanies the protein, and this always gives a higher value compared with the precipitate formed by basic lead acetate.

The difference, therefore, of both determinations might be considered fairly well as the least amounts of the nitrogen of albumoses and peptones. Further, if we recalculate the nitrogen contents mentioned above, assuming the total nitrogen as 100, we will get a clearer conception about the fact.

	Sample obtained by B. No. 34. yeast.	Sample obtained by A. 21. variety.
Total-N.	100.00	100.00
Protein-N.		
a) STUTZER'S method.	47.66	45.36
b) RÜMPLER'S method.	23.69	22.85
c) Basic Pb-acetate ppte.	8.37	9.39
Difference of a) and c).....	39.29	35.97
Difference of b) and c).....	15.32	13.46

A further research was carried on to determine separately the nitrogen of albumoses and peptones. Thus, the filtrate from basic-lead acetate was treated with H_2S and the filtrate from sulphide was evaporated to a small volume and after acidifying with sulphuric acid the solution was saturated with zinc sulphate to precipitate the *albumoses*. The filtrate from the albumoses was treated by a current of H_2S gas to remove the zinc, and the

1. SÖRENSEN'S Formal method.

filtrate obtained therefrom was evaporated to a small volume. *Peptones* were precipitated from this solution by phosphotungstic acid in acidifying with sulphuric acid. The results are as tabulated below:—

Samples obtained by B. No. 34. variety.		Samples obtained by A. No. 21. variety.	
Albumoses-N.	0.00487 grms. in 100 c.c.	0.00377 grms. in 100 c.c.	
Peptones-N.	0.02666 grms. ,,	0.02174 grms. ,,	
	0.03152 grms. ,,	0.02551 grms. ,,	

Thus, the contents of albumoses and peptones in the sample of the first column in the table are evidently greater than that of the second column, conforming with the result of the determination already quoted.

The writer is inclined to conclude that the property of the "head" of saké-mash has a great influence on the quality of saké, and that the cause of high and thick "head" must be ascribed to the richness in albumoses and peptones in it.

The Change of Amino-acids of Saké during its Storage in Summer, and the Discovery of Means to foresee the Disease of Saké.

BY

Teizō Takahashi.

With Plates I—VII.

How the storage of saké can be improved is at present a problem in the industry of saké brewing in Japan; for the incompleteness of the system of storage troubles us very often, as it is the cause of a saké disease, which is called "Hyochi."

The vat which is used for the storage of saké is nothing more than a common fermenting vat with wooden covers, made from the wood of *Cryptomeria japonica*, from which a characteristic flavour goes into the saké, and almost all people who are accustomed to drink this beverage agree in the requirement of this flavour to the drink. The difficulties of the improvement of the apparatus and thereby the manner of storage consists chiefly in this fact and we are obliged, at least at present, to keep as much as possible the present system of storage.

The writer has already reported¹ on the occurrence of some varieties of *Willia anomala*, which assist very favourably in the ripening of saké during the storage. A property which distinguishes these varieties from common saké yeast is, among others, their greater assimilability of amino-acids compared with that of saké yeast. On the other hand, in investigating the fermentation products of pure "Hyochi" bacilli in saké, there was found as an almost constant change, an increase of amino-acids. These two counter

1. Jour. of Coll. of Agric. Tokyo. Imp. Univ. Vol. I, No. 3. p. 228.

[Jour. Coll. Agric., Vol. V, No. 2, 1913]

acting properties of these two groups of microbes induced me to investigate the change of the amino-acids in saké from time to time during storage, in anticipation that we thus might foresee the disease of saké or "Hyochi" by this investigation; for during the ripening stage of saké, these two groups of microbes must naturally, almost in every case, be present alive and acting quite antagonistically in respect to the alteration of amino-acids of saké.

The method applied for the testing of saké for amino-acids was SÖRENSEN'S Formal method, and the result tolerably justified the assumption that the "Hyochi" could be detected at a very early stage.

I. Preliminary Experiment.

For a preliminary observation, the estimation of amino-acids was carried on with samples contained in flasks taken from the storage vats and stored in a warmer place than the storage room. These samples, being small in quantity and placed in a warmer room, would deteriorate naturally quicker than the beverage in the vats, so in certain cases these samples can be taken as an indicator of the alteration of the drink in the vats; but this mode of foreseeing the disease is very imperfect, having many exceptions. The chief cause of the imperfection of this foreseeing is a lack of scientific observation; for a possible alteration is solely determined by the flavour of the beverage.

The samples observed were 332 in number, obtained from several localities in Japan, and half of them were taken from the storage vats, the other half from the flasks corresponding to the beverage of each vat.

Table I.

Number of Vat.	10/VII. Samples from Vat. Amino-acids. %.	14/VII. Same saké in flask, sampled 25 days previously. Amino-acids. %.	Increase or Decrease. %.	Remarks.
169	0.2246	0.2245	—	{ Sample in flask was already in "Hyochi" stage.
118	0.2278	0.2321	+0.0043	
173	—	0.2324	—	
200	0.2013	0.2049	+0.0036	
56	0.1920	0.2101	+0.0011	

Table II.

Number of Vat.	11/VII.	16/VII. sampled 28 days before.	Increase or Decrease.	Remarks.
54	0.2882	0.3212	+0.0330	{ Sample in flask was already in "Hyochi" state.
25	0.2893	0.2816	-0.0077	
35	0.2593	0.2806	+0.0213	
107	0.1782	0.2976	+0.1193	

Table III.

Number of Vat.	14/VII.	14/VII. sampled 9 days before.	Increase or Decrease.	Remarks.
124	0.2134	0.2112	-0.0022	{ Sample in flask was already in "Hyochi" stage.
14	0.1815	0.1859	+0.0044	
122	0.1790	0.1705	-0.0089	
37	0.1835	0.2017	+0.0182	

Table IV.

Number of Vat.	15/VII.	15/VII. sampled 10 days before.	Increase or Decrease.	Remarks.
108	0.1619	0.1867	+0.0248	Sample in flask was already in "Hyochi" stage. " " " " "
77	0.1992	0.2379	+0.0387	
78	0.2003	0.1804	+0.0199	
65	0.2091	0.2117	+0.0126	
85	0.2112	0.2272	+0.0161	

Table V.

Number of Vat.	14/VII. Sample from Vat. Amino-acids. %.	14/VII. Same saké in flask, sampled 9 days before. Amino-acids. %.	Increase or Decrease. %.	Remarks.
47	0.1958	0.2178	+0.022	Sample in flask was in "Hyochi" stage.

Table VI.

Number of Vat.	20/VII.	20/VII. sampled 5 days before.	Increase or Decrease.	Remarks.
1	0.201	0.2152	+0.0151	
3	0.198	0.2280	+0.030	
5	0.1845	0.2518	+0.0313	
6	0.1866	0.2170	+0.0304	
7	0.1965	0.2192	+0.0227	
9	0.1934	0.2262	+0.0328	

10	0.2019	0.2171	+0.0155
11	0.2208	0.2031	+0.0177
12	0.2034	0.1923	-0.0111
13	0.2055	0.2185	+0.013
14	0.1885	0.2105	+0.022
15	0.1960	0.2210	+0.025
16	0.2107	0.1974	-0.0133
17	0.2028	0.2160	+0.0132
18	0.2027	0.1878	+0.1878

Table VII.

Number of Vat.	21/VII.	21/VII. sampled 6 days before.	Increase or Decrease.	Remarks.
22	0.2373	0.2120	-0.0253	
24	0.2077	0.2177	+0.0100	
25	0.2130	0.2054	-0.076	
27	0.2082	0.1741	-0.0341	
28	0.2270	0.2220	-0.005	
29	0.2172	0.2156	-0.0016	
35	0.2320	0.289	+0.037	

Table VIII.

Number of Vat.	21/VII. sample from vat. Amino-acids. %.	21/VII. sampled 14 days before. Amino-acids. %.	Increase or Decrease.	Remarks.
1	0.1515	0.1550	+0.0035	
2	0.1605	0.1750	+0.0145	
3	0.1730	0.1640	-0.0090	
5	0.1716	0.1941	+0.0225	
6	0.1726	0.1693	-0.0033	
7	0.1759	0.1743	-0.0016	
8	0.1753	0.1628	-0.0125	
9	0.1717	0.1730	+0.0013	
10	0.1682	0.1197 ?	-0.0485 ?	
11	0.1669	0.1720	+0.0051	
13	0.1810	0.1587	-0.0223	
14	0.1810	0.1735	-0.0075	
15	0.1760	0.1766	+0.0006	
16	0.1830	0.1898	+0.0068	
17	0.1880	0.1703	-0.0177	
19	0.1710	0.1630	-0.0080	
24	0.1692	0.1989	+0.0297	
26	0.1640	0.1580	-0.0070	
27	0.1770	0.1647	-0.0123	

Table IX.

Number of Vat.	21/VII.	21/VII. sampled 25 days before.	Increase or Decrease.	Remarks.
8	0.2140	0.1999	-0.0141	
11	0.2143	0.2370	+0.0227	

12	0.2120	0.2130	+0.0010
14	0.1971	0.1610	-0.0361
17	0.1980	0.1990	+0.001
18	0.2044	0.1992	-0.0152
19	0.1985	0.2159	-0.0174

Table X.

Number of Vat.	22/VII.	22/VII. sampled 26 days before.	Increase or Decrease.	Remarks.
25	0.2138	0.2010	-0.0128	
26	0.2140	0.2216	+0.0076	
27	0.2208	0.1977	-0.0231	
29	0.2225	0.3543	+0.0218	
30	0.2237	0.2020	-0.0217	
32	0.2035	0.2101	+0.0066	
33	0.1788	0.1836	+0.0048	
34	0.1786	0.1790	+0.0004	
36	0.1811	0.1849	+0.0038	
37	0.1811	0.1849	+0.0038	
44	0.1912	0.2005	+0.0093	
48	0.1733	0.1824	+0.0041	
49	0.1841	0.1889	+0.0048	
50	0.1874	0.1927	+0.0053	
53	0.1919	0.2089	+0.0170	
54	—	0.1888	—	

Table XI.

Number of Vat.	1/VIII.	1/VIII. sampled 8 days before.	Increase or Decrease.	Remarks.
3	0.2240	0.2234	-0.0006	
4	0.2008	0.2122	+0.0114	
5	0.2105	0.2090	-0.0015	
6	0.2014	0.1894	-0.0120	
7	0.2381	0.2384	-0.0005	
8	0.2439	0.2483	+0.0044	

Table XII.

Number of Vat.	2/VIII.	2/VIII. sampled 9 days before.	Increase or Decrease.	Remarks.
9	0.2322	0.2395	+0.0073	
10	0.2196	0.2099	-0.0097	
11	0.1871	0.1533	-0.0338	
12	0.2409	0.2617	+0.0208	

Table XIII.

Number of Vat.	1/VIII.	1/VIII. sampled 9 days before.	Increase or Decrease.	Remarks.
15	0.204	0.209	+0.005	Sampled 15 days before. 0.220 0.219
16	0.219	0.231	+0.012	

Table XIV.

Number of Vat.	2/VIII.	2/VIII. sampled 10 days before.	Increase or Decrease.	Remarks.
17	0.247	0.245	-0.0020	
18	0.242	0.249	+0.007	
19	0.229	0.212	-0.017	
24	0.231	0.214	-0.017	
25	0.214	0.214	—	
26	0.221	0.216	-0.005	
27	0.224	—	—	
28	0.214	0.225	+0.011	
29	0.205	0.207	+0.002	
30	0.217	0.204	-0.013	
31	0.268	0.273	+0.005	
33	—	0.195	—	

Table XV.

Number of Vat.	6/VIII.	12/VIII. sampled 5 days before.	Increase or Decrease.	Remarks.
8	0.2202	0.2219	+0.0017	
42	0.2205	—	—	
46	0.2236	0.2169	-0.0067	
70	0.2247	0.2260	+0.0014	
75	0.2274	0.2391	+0.0117	
101	0.2261	0.2244	+0.0017	
112	0.2172	0.2292	+0.0120	
140	0.2182	0.2292	+0.0110	
141	0.2187	0.2292	+0.0105	
180	0.2195	0.2317	+0.0122	
181	0.2325	0.2406	+0.0081	
186	0.2219	0.2325	+0.0106	
290	0.2466	0.2717	+0.0251	
301	0.2357	0.2488	+0.0131	
305	0.2292	0.2406	+0.0114	
307	0.2514	0.2660	+0.0146	
311	0.2336	0.2586	+0.0250	
319	0.2257	0.2391	+0.0134	
321	0.2185	0.2456	+0.0271	
322	0.2432	0.2720	+0.0288	

Table XVI.

Number of Vat.	6/VIII.	8/VIII. sampled 24 days before.	Increase or Decrease.	Remarks.
34	0.2833	0.2940	+0.0107	
37	0.3025	0.3161	+0.0136	
38	0.2966	0.2817	-0.0151	

Table XVII.

Number of Vat.	6/VIII.	9/VIII. sampled 23 days before.	Increase or Decrease.	Remarks.
35	0.3096	0.3242	+0.0146	
42	0.2854	0.3044	+0.0190	

Table XVIII.

Number of Vat.	7/VIII.	7/VIII. sampled 8 days before.	Increase or Decrease.	Remarks.
251	0.2464	0.2443	-0.0021	
350	0.1937	0.2043	+0.0105	
360	0.2114	0.2122	+0.0008	
192	0.3283	0.3183	+0.01000	
226	0.2522	0.2653	+0.0131	
366	0.2229	0.2262	+0.0033	
344	0.2378	0.2449	+0.0078	

Table XIX.

Number of Vat.	7/VIII.	7/VIII. sampled 6 days before.	Increase or Decrease.	Remarks.
248	0.2467	0.2616	+0.0149	
15	0.2838	0.2856	+0.0018	
41	0.2089	0.2177	+0.0087	
250	0.2688	0.2838	+0.0150	
263	0.2188	0.2326	+0.0138	
265	0.2022	0.2091	+0.0069	
298	0.2255	0.2368	+0.0113	

The increase of amino-acids is observable in almost every sample of the flask, but the degree of increase of amino-acids must naturally vary in accordance with the quality of saké or the varieties of "Hyochi" bacilli, by which the saké was contaminated, so in certain cases there was found an increase of 0.037% during six days, or 0.033%, or 0.1195% in 28 days; while in other cases only a very small increase, such as 0.0011% was found even after 25 days. The antagonistic behaviour of *Willia anomala* to "Hyochi" bacilli was met with in certain samples which showed, in an extreme case, a decrease of amino-acids amounting to 0.0223%.

Above all, the most interesting fact is that this increase of amino-acids was certain, even when there was no perceptible symptom of "Hyochi," and this must be taken as an advance of the method to that of already known of foreseeing "Hyochi."

II. The Observation of the Change of Amino-acids in the Storage Vat.

In the preliminary experiment the expectation was ascertained tolerably well, so some further researches were made with saké in the storage vats.

The samples were taken from time to time from the storage vat and analysed at the factory in which the samples were kept. The number of the factories supplying the samples was 36, located chiefly in the western part of Japan. The storage vats from which samples were drawn amounted to 533 in number, but only some of them will be tabulated here:—

Table XX.

Numb. of Vat.	13/VII.	8/VIII.	25/VIII.	9/IX.	22/X.	Remarks.
11	0.2073	0.2233	0.1977	0.2000	0.2121	{ On 8/X a heavy turbidity was observed in the sample. On 1/X "Hyochi" flavour was ascertained.
24	0.2426	0.2566	0.2533	0.2633	0.2697	
67	0.2243	0.2266	0.2240	0.2200	0.2363	
127	0.2090	0.2133	0.2077	0.2100	0.2424	{ On 20/IX a slight turbidity and "Hyochi" flavour was notice- able.
125	0.2516	0.2361	0.2406	0.2433	0.2454	
57	0.2563	0.2833	0.2636	0.2666	0.2818	
84	0.2365	0.2266	0.2270	0.2266	0.2242	{ The best sample in quality in the factory.
81	0.2123	0.2266	0.2170	0.2167	0.2273 _(5/XI)	
124	0.2089	0.2000	0.2020	0.2000	0.2212	
123	0.2433	0.2433	0.2373	0.2366	0.2424	{ On 20/X "Hyochi" flavour was ascertained.
21	0.2300	0.2533	0.2463	0.2333	0.2424	
19	0.1827	0.2057	0.2060	0.2067	0.2121	
63	0.2603	0.2663	0.2663	0.2360	0.2818	{ On 24/VIII "Hyochi" flavour was ascertained.
13	0.2283	0.2396	0.2326	0.2300	0.2333	
10	0.2113	0.2263	0.2057	0.2060	0.2121	
60	0.2295	0.2496	0.2476	0.2388	0.2515	{ On 24/VIII "Hyochi" flavour was ascertained.
78	0.2310	0.2463	0.2060	0.2242	0.2454	
65	0.2376	0.2400	0.2446	0.2666	0.2666	
111	0.2310	0.2633	0.2493	0.2497	0.2524	{ On 24/VIII "Hyochi" flavour was ascertained.
119	0.2193	0.2530	0.2300	0.2288	0.2485	

Table XXI.

Numb. of Vat.		6/VIII.	25/VIII.	26/IX.	4/XI.	Remarks.
52	0.2313	0.2396	0.2373	0.2485	0.2363	{ On 8/IX the flavour of "Hyochi" was ascertained. On 8/IX began to become tur- bid and "Hyochi" flavour followed.
55	0.2313	0.2193	0.2173	—	0.2363 _(5/XI)	
58	0.1987	0.2360	0.2240	0.2379	0.2366 _(5/XI)	
56	0.2313	0.2326	0.2173	0.2303	0.2400	{ On 24/VIII "Hyochi" flavour was ascertained.
67	0.1913	0.1956	0.2040	0.2071	0.1970	
66	0.2146	0.2326	0.2240	0.2303	0.2151	
80	0.2053	0.2123	0.2110	0.2273	—	{ On 24/VIII "Hyochi" flavour was ascertained.
68	0.1950	0.2360	0.2203	0.2257	0.2121	
49	0.2020	0.1986	0.1973	0.2136	0.2030	
82	0.2083	0.2093	0.1943	0.1970	0.1934	{ On 24/VIII "Hyochi" flavour was ascertained.
71	0.2180	0.2326	0.2273	0.2363	0.2332	
65	0.2280	0.2296	0.2170	0.2273	0.2182	
73	0.2033	0.2326	0.2093	0.2106	0.2121	{ On 24/VIII "Hyochi" flavour was ascertained.
51	0.2413	0.2393	0.2260	0.2363	—	
50	0.2020	0.2363	0.2293	0.2182	—	
72	0.2410	0.2496	0.2326	0.2424	0.2333	{ On 24/VIII "Hyochi" flavour was ascertained.
92	0.2336	0.2533	0.2260	0.2427	0.2324	
70	0.2283	0.2423	0.2360	0.2439	0.2303	
69	0.2473	0.2493	0.2360	0.2394	0.2333	{ On 24/VIII "Hyochi" flavour was ascertained.

86	0.2060	0.2260	0.2240	0.2318	0.2182	
64	0.2476	0.2426	0.2326	0.2363	0.2333	
91	0.2383	0.2360	0.2330	0.2439	0.2182	
48	0.2450	0.2493	0.2343	0.2539	0.2273	
53	0.2170	0.2496	0.2266	0.2469	—	
90	0.2413	0.2533	0.2530	0.2348	0.2273	
67	0.2303	0.2293	0.2240	0.2348	0.2303	

Table XII.

Numb. of Vat.	1/VIII.	17/VIII.	5/IX.	27/IX.	11/X.	Remarks.
2	0.2086	0.2320	0.2280	—	—	“Hyochi.”
3	0.2240	0.2310	0.2190	0.2240	0.2410	
4	0.2008	0.2140	0.2160	0.2150	0.2220	
5	0.2105	0.2120	0.2100	0.2170	0.2300	
6	0.2014	0.2150	0.2130	0.2120	0.2130	
7	0.2381	0.2480	0.2470	0.2500	0.2740	
8	0.2439 ₂ /VIII	0.2420	0.2490	0.2460	0.2730	
9	0.2322 „	0.2270	0.2390	0.2410	0.2610	
10	0.2196 „	0.2380	0.2430	0.2410	0.2440	
11	0.1871 „	0.2050	0.2180	0.2150	0.2440	
12	0.2409 ₁ /VIII	0.2450	0.2640	0.2560	0.2750	
14	— „	0.2280	0.2320	—	—	“Hyochi” on the beginning of October.
15	0.2040 „	0.2200	0.2370	0.2430	—	
16	0.2190 „	0.2030	0.2150	0.2220	0.2320	
17	0.2470 „	0.2310	0.2410	0.2490	0.2590	
18	0.2420 „	0.2300	0.2390	0.2470	0.2550	
19	0.2290 „	0.2080	0.2150	—	—	
24	0.2310 „	0.1960	0.2220	0.2220	0.2260	
25	0.2140 „	0.1890	0.2080	0.2020	0.2020	
26	0.2210 „	0.2090	0.2110	0.2010	0.2150	
27	0.2240 „	0.2100	0.2120	0.2080	0.2090	
28	0.2140 „	0.2090	0.2110	0.2010	0.2150	
29	0.2050 „	0.2050	0.2060	0.2060	0.2080	
30	0.2170 „	0.2030	0.2100	0.2100	0.2100	
31	0.2680 „	0.2350	0.2470	0.2580	0.2780	
33	—	0.2180	0.2210	—	—	

Table XXIII.

Numb. of Vat.	20/VII.	4/VIII.	19/VIII. sampled on 18/VIII.	9/IX.	1/X.	Remarks.
1	0.2010	0.1771	0.1774	0.1723	—	On 2/X “Hyochi” flavour was perceived.
3	0.1980	0.1809	0.1886	0.1869	0.1950	
5	0.1845	0.1782	0.1801	0.1758	0.1836	
6	0.1866	0.1717	—	—	—	On 7/VIII “Hyochi” flavour was perceived.
7	0.1965	0.1687	0.1724	0.1652	—	
9	0.1934	0.1719	—	—	—	
10	0.2019	0.1769	0.1858	0.1816	—	On 18/IX “Hyochi” flavour was perceived.
11	0.2208	0.1695	0.1835	0.1801	0.1866	
13	0.2055	0.1785	0.1840	0.1824	0.1907	
14	0.1883	0.1672	0.1755	0.1713	0.1727	
16	0.2107	0.1854	0.1870	0.1873	0.1915	
18	0.2027	0.1725	0.1706	0.1703	0.1648	
22	0.2373	0.1983	0.1946	0.1903	0.1900	
24	0.2077	0.1827	0.1784	0.1713	—	

25	0.2130	0.1941	—	—	—	On 8/VIII, "Hyo-chi" flavour was perceived.
27	0.2082	0.1763	0.1769	0.1662	—	
28	0.2270	0.1911	—	—	—	
35	0.2320	0.1830	0.1882	—	—	

Table XXIV.

Numb. of Vat.	6/VIII.	19/VIII.	2/IX.	16/IX.	4/X.	23/X.	Remarks.
8	0.2202	0.2236	0.2114	0.2179	0.2316	0.2365	
42	0.2205	0.2240	0.2292	0.2252	0.2259	0.2268	
46	0.2236	0.2147	0.2082	0.2066	0.2219	0.2187	
70	0.2247	0.2309	0.2106	0.2187	0.2038	0.2308	
75	0.2274	0.2381	0.2281	0.2366	0.2243	0.2308	
101	0.2261	0.2098	0.2130	0.2163	0.2187	0.2154	
112	0.2172	0.2228	0.2130	0.2203	0.2349	0.2349	
140	0.2182	0.2276	0.2184	0.2171	0.2268	0.2292	
141	0.2187	0.2292	0.2226	0.2163	0.2195	0.2187	
180	0.2195	0.2268	0.2203	0.2179	0.2255	0.2292	
181	0.2325	0.2244	0.2187	0.2187	0.2308	0.2365	
186	0.2219	0.2163	0.2106	0.2114	0.2187	0.2211	
290	0.2466	0.2600	0.2524	0.2730	0.2705	—	
301	0.2257	0.2430	0.2244	0.2487	0.2454	0.2413	
305	0.2292	0.2244	0.2236	0.2219	0.2292	0.2324	
307	0.2514	0.2516	0.2487	—	—	—	
311	0.2336	0.2454	0.2273	0.2354	0.2470	0.2422	
319	—	0.2362	0.2187	0.2211	0.2235	0.2357	
321	0.2185	0.2341	0.2211	0.2211	0.2300	0.2340	
322	0.2432	0.2702	0.2754	0.2681	0.2664	0.2656	

Table XXV.

Numb. of Vat.	7/VIII.	20/VIII.	3/IX.	17/IX.	5/X.	24/X.	Remarks.
251	0.2464	0.2454	0.2324	0.2197	0.2478	0.2543	
350	0.1937	0.2008	0.1925	0.2008	0.2008	0.1984	
360	0.2114	0.2162	0.2057	0.2211	0.2178	0.2130	
192	0.3283	0.8223	0.3340	0.3223	0.3369	—	
226	0.2522	0.2511	0.2494	0.2705	0.2705	0.2713	
366	0.2229	0.2373	0.2227	0.2268	0.2202	0.2154	
344	0.2378	0.2373	0.2316	0.2259	0.2381	0.2389	On 14/VIII "Hyo-chi" flavour was perceived.

The quantity of amino-acids of sample No. 119 on the first table, one of the best beverages at this factory, showed a decrease at every determination from 9th August until 8th Sept.; while No. 10, another of the best samples, showed an increasing disposition already on 9th Sept., and in the third best sample, No. 19, the quantity increased from the beginning of the experiment (13th July) until 9th Sept., though the degree of the increase was very small in the last two determinations. Thus, the alteration of the quantity of amino-acids is altogether indifferent to the quality of saké, inasmuch as

the former sample showed an enormous change from 13th July to 9th Sept., while the latter two changed relatively in a smaller degree. Besides this, in Plate I the interesting fact is observed that, in general, the minimum quantity of amino-acids in all the samples, with the exception of two, are shown on the determinations of 25th Aug. and 9th Sept., i.e. at the end of Aug. or in the beginning of September. As I have pointed out in the former research,¹ the best sort of saké contains the least quantity of amino-acids, the stage in which the minimum quantity of it is contained, is the most favourable time with respect to the quality of the drink. *From this point of view, the ripening of saké should, perhaps, be stopped at the end of August or in the beginning of September.*

The samples which have suffered from bacilli or been affected by "Hyochi" (No. 78, 67, 81), want special notice, inasmuch as they have at first shown *special or continued decrease of amino-acids* before they deteriorated, and this is evidence of the function of the so-called aging yeast, *Willia anomala*, which assimilates amino-acids specially well. However, the activity of the aging yeast must cease sooner or later as soon as the organism attains its full growth, and subsequently to its resting stage, giving place to "Hyochi" bacilli. This is the reason, why we must pay special care to those vats in which a decrease of amino-acids occurs. From this point of view, the analysis of 9th September gave notice of danger for the samples No. 78, 67, and 81, which would no doubt have been confirmed if the experiment had been repeated a few days later with the latter two samples. The samples No. 21, 60, and 63 are other examples which want special care for their further storage; for they have shown *two continued decreases of amino-acids*, especially No. 63, in the analysis on 25th Aug. and 9th Sept. Other examples which must be included in the same category are No. 57, 65, and 11. Their decrease in the quantity of amino-acids is very conspicuous on 25th Aug., when compared to that of 8th of the same month; they are surely destined to suffer from the damage of "Hyochi," though they were still sound on the 22nd of October. The samples No. 84, 123, 124 and 125 are marked in the plate with *blue lines*

1. TEIZÔ TAKAHASHI and HISAE SATÔ, Journal of the College of Agric. Tokyo Imp. Univ., Tokyo. Vol. I. No. 3.

to show that they remained sound at the end of the observation and the degree of alteration of the quantity of amino-acids is relatively small.

In the next plate (Pl. II, III), three lines of three altered brewings are shown. They must be included in the same category as the altered samples of the first plate, namely, they had fallen first to their minimum quantities of amino-acids before they suffered damage by "Hyochi." Other samples remained sound at the end of the observation, and all of them expressed relatively a smaller degree of alteration in the quantities of amino-acids, especially the samples No. 65 and 82 (Pl. II).

Sample No. 90. gave to the plate a special line, for it shows two continued increases (6th and 25th Aug.) and two successive decreases in the quantity of amino-acids, so perhaps, the last amounts were indications that the danger of damage was very near, but fortunately the coolness of the season kept the sample sound.

In the samples, which are represented on Plate IV, only one of them (No. 15) suffered from the damage. It gave two successive and relatively higher degrees of increase in the amount of amino-acids and in the third experiment it was "Hyochi" saké already. Perhaps, the minimum amounts of amino-acids had passed already at the beginning of the observation, and if this was not the case, the sample is one which was destined to suffer the damage from the beginning of the storage.

Another set of samples (No. 9, 11, 12, 24, 25, 31), though they kept sound until the last examination, their changes in the amount of amino-acids were very conspicuous, especially so with No. 31, showing three successive increases of the acids. They were sure to go bad in the near future, the former three samples (No. 9, 11, 12) are very interesting; for they showed an enormous change during ten days in the preliminary experiment, especially so No. 9, which was in "Hyochi" stage already at the end of the ten days.

About Plate V the writer could find no factor for foreseeing the "Hyochi" disease; because the experiment was commenced too late for this purpose.

In the samples represented on Plate VI, there is not one which has developed the "Hyochi" damage, but an interesting fact is that the results

of the preliminary experiments tell us quite certainly the future uncertainty of the beverage in the vat. *The red dotted lines in the plate indicate alarm of damage as the result of the experiments.* Almost the same fact is represented in Plate VII, especially with No. 226.

Summary.

The changes of the amounts of amino-acids of saké in the storage vats are relatively large, showing very clearly the alternative increase and decrease of them. It is highly probable that these changes are caused by the co-existence of the aging yeast, *Willia anomala* and "Hyochi" bacilli active and acting quite antagonistically i.e. in the state of the struggle for existence.

The damage of so-called "Hyochi" is certain to follow when the quantity of amino-acids always increases at successive examinations, or after successive decreases of the acids are observed, especially when the minimum point is reached at a time which is yet too warm to depress the growth of the "Hyochi" bacilli. Therefore we must notice the degree of the decrease of the acid by reason of the most active growth of the aging yeast, which at the end of its activity will be followed by the growth of the "Hyochi" bacilli.

Sometimes, the changes in the storage vats are foreseen from the changes in the preliminary experiment with the samples, specially if taken in the flask and kept in a warmer place for a few days.

Generally, the beverage which keeps sound for a relatively long time, shows very small changes in the amounts of amino-acids.



of the 21 samples of Moto rice contain above 14% water. 10 samples of the 23 of Kaké rice contain less than 13% water, and only one of Kaké rice contains over 14% water. It therefore appears that the water content of Kaké rice is generally less than that of Moto rice; this point seems to call for further observation.

As regards the weight per 1.8 liters, we see that *better rice is heavier than inferior*, and also that the length, breadth and thickness of the grains of better rice are larger than those of inferior kinds. If Moto rice and Kaké rice obtained from the same district be compared, the former is always heavier than the latter. To what this difference is due, whether to chance or to the degree of whitening, or whether the manufacturers as a result of their long experience unconsciously choose such a kind of rice, has not been determined. When calculated for dry matter we obtain results as in Table II.

From Table II we see that the sugar content of the majority ranges from 0.5% to 0.7%, the minimum and maximum being 0.505% and 0.787%. It is also clear that the sugar content has no relation to the localities which produced the rice; but there exist differences according to the localities even in the same varieties. The content of fat differs according to varieties and localities, the minimum being 0.2364% and the maximum 1.1822%. The more the loss by whitening the less is the quantity of fat, provided the varieties of rice and districts where they were grown be the same. The total nitrogen content ranges from 1.3% to 1.4%. As there is hardly any difference in the quantities of nitrogen between well whitened rice of better quality and that of common quality whitened to a less degree, it follows that if both were whitened to the same degree the former would contain comparatively more protein than the latter. Of the quantity of non-albuminoid nitrogen, the minimum is 0.1102% and the maximum 0.3574%. Of the quantity of ash, the minimum is 0.2948% and the maximum 0.3805%. Some of the rices of better quality especially grown in Settsu and Harima prefectures contain generally more ash compared to others of inferior quality. Of potash and phosphoric acid, which make up the greater part of the ash, no special relation can be perceived. According to the analysis of Y. KOZAI and H. ANDO of the ash ingredients of rice, the quantity of iron varies according to the district which produced the rice.

Our results also show the same fact, i. e. Korean and Akita rice contained more iron than others. The quantity of iron decreases as the process of whitening is carried further.

II. Proteins.

Two kinds of rice were used for the investigation; No. 1 was Moto rice with a loss by whitening of 24%, and the other No. 2 was Kaké rice with a loss of 17%. Their general chemical composition was as follows (samples first washed well then air dried and analysed) :

	No. 1 (Moto rice)	No. 2 (Kaké rice)
Water	14.567	14.643
Fat	0.280	0.360
Starch	76.086	74.883
Sugar	0.460	0.240
Dextrin	1.058	0.852
Total nitrogen	1.268	1.312
Albuminoid nitrogen	1.106	1.2006
Non-albuminoid nitrogen	0.162	0.112
Cellulose	0.307	0.404
Ash	0.290	0.208
Phosphoric acid	0.141	0.120

Proteins were isolated chiefly according to OSBORNE'S method.

The whitened rice was well washed with water, then completely air dried and finely powdered. 5 kilo of each was soaked in 15 litres water for 24 hours, then filtered, the filtrate evaporated (during the evaporation flocculum was observed in No. 2) at low temperature to a small volume, precipitated with alcohol, and the precipitate dried. The rice treated as above was again soaked in 15 litres of 10% sodium chloride solution for 24 hours, filtered, and the filtrate evaporated to a small volume, saturated with ammonium sulphate, dialysed until the filtrate gave no reaction for chlorine, then evaporated to dryness at 70° C or below. After this second treatment the residue was again soaked in 15 litres of 0.2% *Na OH* solution during 24 hours, filtered, the filtrate neutralized with acetic acid to faint acid

reaction, the precipitate obtained gathered on filter papers, well washed with water, alcohol and ether, and then dried.

Another 5 kilo of newly prepared rice was soaked in 15 litres of 80% alcohol for 24 hours filtered, the filtrate evaporated to dryness and the fat was extracted with anhydrous ether. After the alcohol had been expelled from the residue treated as above, it was again soaked in 15 litres of 0.2% soda-lye solution during 24 hours, filtered, the filtrate neutralized with acetic acid to slight acid reaction, the precipitate obtained gathered on filter papers, well washed with water, alcohol, and ether and dried.

OSBORNE'S method¹ for the preparation of proteins soluble in 80% alcohol was tried for rice flour. The somewhat concentrated extract was poured in a very fine stream into ice water, but no precipitate formed, only some turbidity was to be seen. Thereupon the beaker containing the ice water was immersed in calcium chloride solution at -6°C and left for 17 hours, but no precipitate could be found.

Four kinds of proteins were obtained by the methods described above; viz. albumin, a protein soluble in water, globulin, a protein soluble in 10% sodium chloride solution, prolamin, a protein with bitter taste, soluble in 80% alcohol, and glutenin:—ROSENHEIM'S oryzenin, a protein soluble in a dilute alkaline solution. These were subjected to MILLON'S, biuret, and ADAM-KIEWICZ'S reaction, (tryptophane test according to HOPKINS and COLE'S modification), which all gave positive results, while with the last proof showing a somewhat different shade in color, as follows:—

	No. I (Moto rice)	No. II (Kake rice)
Albumin.....	blue	blue
Globulin.....	blue	blue
Prolamin	greenish red or dirty green	green
Oryzenin.....	blue	blue

Test of Iron.—Each of these four proteins was digested with hydrochloric acid, and with the exception of the albumin, they all gave prussian-blue reaction. It has been proved by U. SUZUKI that iron exists in rice in combination with protein.

1. The Americ. Jour. Physiol. Vol. XX. Jan. 1908 No. 1 c. Vol. XX, No. 4. p. 496 (the hydrolysis of gliadin from rye) and The Jour. of the Americ. Chem. Soc. Vol. XVII, No. 7. July 1895. p. 557.

Test of sulphur.—A sample of each of the proteins was heated with metallic sodium and plunged into water as usual. The solutions were treated with nitroprussid sodium or lead acetate with acetic acid; the results were as follows:—

	Sample No. 1		Sample No. 2	
	Nitroprussid sodium	Lead acetate + Acetic acid	Nitroprussid sodium	Lead acetate + Acetic acid
Albumin	Greenish yellow	Colorless ppt.	Greenish yellow	Colorless ppt.
Globulin	Purple, next reddish brown, next yellow	Black ppt.	Thin purple, red. greenish yellow, yellow	Brown ppt.
Prolamin	Thin purple, reddish brown, thin yellow	Brown ppt.	Thin purple, then same as above	Brown ppt.
Oryzenin	Purple, yellowish brown, brownish yellow	Black ppt.	Purple, then same as above	Black ppt.

The proteins were then subjected to elementary analysis, and though the water extract contained too much carbohydrates, the nitrogen was determined according to DUMA's method:—

	Water ext. No. 1.	Water ext. No. 2.
Nitrogen.....	0.60%	0.33%
Dextrin	53.82%	63.92%
Glucose	9.80%	4.00%

The result of the elementary analysis of the *flocculum*, albumin, obtained from No. 2 water extract, and the othert hree kinds of proteins were as follows (as the samples contained sulphur lead chromate was used for incineration).

The albumin obtained by heating the No. 2 water extract at 60-70° C.

a) 0.1869 gr. used.

0.3620 gr. carbon dioxide gas. 55.7% carbon.
0.1400 gr. water. 9.3% hydrogen.

b) 0.1523 gr. used.

20.6 c.c. nitrogen gas at 25° C and 765 m.m.
15.9% nitrogen.

20.1% oxygen and sulphur.

Globulin No. 1 sample.

a)	0.1714 gr.	used.		
	0.2780 gr.	carbon dioxide gas.	44.2%	carbon.
	0.1186 gr.	water.	7.6%	hydrogen.
b)	0.1914 gr.	used.		
	0.3070 gr.	carbon dioxide gas.	43.7%	carbon
	0.1258 gr.	water.	7.3%	hydrogen.
			43.95%	carbon.
			7.45%	hydrogen.
			} average.	
c)	0.1134 gr.	used.		
	12.8 c.c.	nitrogen gas at 8.8° C and 755.4 m.m.		
			13.8%	nitrogen.
			33.8%	oxygen and sulphur.

Globulin No. 2 sample.

a)	0.2042 gr.	used.		
	0.3650 gr.	carbon dioxide gas.	48.7%	carbon.
	0.1438 gr.	water.	7.8%	hydrogen.
b)	0.1919 gr.	used.		
	0.3462 gr.	carbon dioxide gas.	49.2%	carbon.
	0.1320 gr.	water.	7.6%	hydrogen.
			48.95%	carbon.
			7.7%	hydrogen.
			} average	
c)	0.1716 gr.	used.		
	22.8 c.c.	nitrogen gas at 10° C and 756 m.m.		
			16.2%	nitrogen.
			27.15%	oxygen and sulphur.

Prolamin No. 1 sample.

	0.1160 gr.	used.		
	13.6 c.c.	nitrogen gas at 9.6° C and 765.7 m.m.		
			14.44%	nitrogen.

Prolamin No. 2 sample.

a)	0.1844 gr.	used.		
	0.3986 gr.	carbon dioxide gas.	58.9%	carbon.
	0.1618 gr.	water.	9.7%	hydrogen.
b)	0.1563 gr.	used.		
	0.3338 gr.	carbon dioxide gas.	58.1%	carbon.
	0.1398 gr.	water.	9.9%	hydrogen.
			58.5%	carbon.
			9.8%	hydrogen.
				} average.
c)	0.0892 gr.	used.		
	10.1 c.c.	nitrogen gas at 11.2° C and 764.2 m.m.		
			13.83%	nitrogen.
d)	0.1106 gr.			
	12.65 c.c.	nitrogen gas at 8.2° C and 758.1 m.m.		
			14.00%	nitrogen.
			13.92%	nitrogen (average).
			17.78%	oxygen and sulphur.

As mentioned above we have two preparations of oryzenin, one prepared after the extraction of globulin without taking off prolamin, and the other prepared after the extraction of prolamin.

Oryzenin No. 1 sample (containing some prolamin).

a)	0.1488 gr.	used.		
	0.3064 gr.	carbon dioxide gas.	56.1%	carbon.
	0.1010 gr.	water.	7.5%	hydrogen.
b)	0.1402 gr.	used.		
	0.2860 gr.	carbon dioxide gas.	55.6%	carbon.
	0.0976 gr.	water.	7.7%	hydrogen.
			55.85%	carbon.
			7.6%	hydrogen.
				} average.

c) 0.1410 gr. used.

16.8 c.c. nitrogen gas at 11° C and 758.5 m.m.

14.5% nitrogen.

d) 0.1718 gr. used.

20.8 c.c. nitrogen gas at 9° C and 758.2 m.m.

14.8% nitrogen.

14.65% nitrogen (average).

21.9% oxygen and sulphur.

Oryzenin No. 2 sample (containing some prolamin).

a) 0.1426 gr. used.

0.2624 gr. carbon dioxide gas. 50.1% carbon.

0.1050 gr. water. 8.1% hydrogen.

b) 0.1284 gr. used.

0.2400 gr. carbon dioxide gas. 50.9% carbon.

0.1074 gr. water. 9.2% hydrogen.

50.5% carbon.	} average.
8.65% hydrogen.	

c) 0.1918 gr. used.

27.2 c.c. nitrogen gas at 10.5° C and 751.5 m.m.

17.1% nitrogen.

d) 0.1882 gr.

26.5 c.c. nitrogen gas at 11.2° C and 758.4 m.m.

17.1% nitrogen.

17.1% nitrogen (average).

23.75% oxygen and sulphur.

Oryzenin No. 1 sample (containing no prolamin).

a) 0.1234 gr. used.

0.2254 gr. carbon dioxide gas. 49.8% carbon.

0.0936 gr. water. 8.4% hydrogen.

b)	0.1648 gr.	used.	
	0.3052 gr.	carbon dioxide gas.	50.5% carbon.
	0.1202 gr.	water.	9.2% hydrogen.
			50.01% carbon.
			8.8% hydrogen.
			} average.
c)	0.1588 gr.	used.	
	22.2 c.c.	nitrogen gas at 10.2° C and 759.2 m.m.	
			17.1% nitrogen.
			24.09% oxygen and sulphur.

Oryzenin No. 2 sample (containing no prolamin).

a)	0.1342	used.	
	0.2440	carbon dioxide gas.	49.5% carbon.
	0.1030	water.	8.5% hydrogen.
b)	0.1644	used.	
	21.1 c.c.	nitrogen gas at 9.5° C and 750.7 m.m.	
			15.5% nitrogen.
			26.5% oxygen and sulphur.

In order to get the proteins in a purer state, 500 gr. each of No. 1 and 2 samples was made into fine powder, first extracted with water several times until the filtrate gave no protein reaction, neither with biuret, ADAM-KIEWICZ's, HOPKINS' and COLE's (tryptophane test). The residue was then treated with 10% sodium chloride solution several times until the filtrate gave no reaction for protein as above. The residue was next extracted with 80% alcohol and lastly with 0.2% *Na OH* solution. During the last extraction a small quantity of toluol was added for the purpose of disinfection, but otherwise the treatment was the same as before.

The quantities of the proteins obtained from 500 gr. of the air dried powder were as follows:—

	Sample No. 1.		Sample No. 2.	
	weight obtained	%	weight obtained	%
Albumin and substances soluble in water	8.13 gr.	1.62	16.00 gr.	3.20
Globulin	2.33 gr.	0.466	During dialysis membrane broken	all lost
Prolamin	2.60 gr.	0.52	2.40 gr.	0.48
Oryzenin	6.20 gr.	1.24	9.80 gr.	1.96

The composition of the water extracts was as follows:—

Water extract No. 1 sample.

Nitrogen, according to Kjeldahl's method 0.252%
(0.252 × 6.25 = 1.575 ∴ as albumin..... 1.575%)

Dextrin 24.12%

Glucose 71.92%

Water extract No 2 sample.

Nitrogen, according to Kjeldahl's method 0.182%
(0.182 × 6.25 = 1.137 ∴ as albumin..... 1.137%)

Dextrin 25.556%

Glucose 69.00%

The remaining three kinds of proteins were subjected to elementary analysis with the following results:

Globulin No. 1 sample.

a) 0.0833 gr. used.

0.1486 gr. carbon dioxide gas. 48.60% carbon.

0.0650 gr. water. 8.6% hydrogen.

b) 0.1696 gr. used.

15.2 c.c. nitrogen gas at 9.7° C and 757.8 m.m.

11.73% nitrogen.

33.09% oxygen and sulphur.

Prolamin No. 1 sample.

a) 0.1838 gr. used.

0.3908 gr. carbon dioxide gas. 58.14% carbon.

0.1470 gr. water. 8.92% hydrogen.

b) 0.1275 gr. used.		
0.2716 gr.	carbon dioxide gas.	58.09% carbon.
0.1038 gr.	water.	9.04% hydrogen.
		48.115% carbon. } average.
		8.98% hydrogen. }
c) 0.1142 gr. used.		
10.8 c.c. nitrogen gas at 26° C and 766.5 m.m.		
10.5% nitrogen.		
d) 0.1503 gr. used.		
14.8 c.c. nitrogen gas at 26.7° C and 766.2 mm.		
10.9% nitrogen.		
		10.7% nitrogen (average).
		22.205% oxygen and sulphur.
Prolamin No. 2 sample.		
a) 0.1574 gr. used.		
0.3464 gr.	carbon dioxide gas.	60.02% carbon.
0.1275 gr.	water.	9.00% hydrogen.
b) 0.1412 gr. used.		
0.3122 gr.	carbon dioxide gas.	60.30% carbon.
0.1194 gr.	water.	9.40% hydrogen.
		60.16% carbon. } average.
		9.20% hydrogen. }
c) 0.1486 gr. used.		
15.2 c.c. nitrogen gas at 28° C and 761.0 m.m.		
11.20% nitrogen.		
d) 0.1694 gr. used.		
16.4 c.c. nitrogen gas at 25° C and 765.8 m.m.		
10.80% nitrogen.		
		11.0% nitrogen (average).
		19.64% oxygen and sulphur.

Oryzenin No. 1 sample.

a) 0.1721 gr. used.

0.3552 gr. carbon dioxide gas. 56.2% carbon.

0.1320 gr. water. 8.5% hydrogen.

b) 0.1785 gr. used.

0.3720 gr. carbon dioxide gas. 56.8% carbon.

0.1300 gr. water. 8.1% hydrogen.

56.5% carbon.	} average.
8.3% hydrogen.	

c) 0.2188 gr. used

27.5 c.c. nitrogen gas at 11.6° C and 762.0 m.m.

15.5% nitrogen.

d) 0.2730 gr. used.

33.9 c.c. nitrogen gas at 10.2° C and 762.4 m.m.

15.1% nitrogen.

15.3% nitrogen (average).

19.9% oxygen and sulphur.

Oryzenin No. 2 sample.

a) 0.1718 gr. used.

0.2637 gr. carbon dioxide gas. 41.8% carbon.

0.1350 gr. water. 8.1% hydrogen.

b) 0.1800 gr. used.

24.4 c.c. nitrogen gas at 12.2° C and 760.7 m.m.

16.4% nitrogen.

c) 0.1890 gr. used.

25.2 c.c. nitrogen gas at 11.2° C and 762.1 m.m.

16.3% nitrogen.

16.35% nitrogen (average.)

33.75% oxygen and sulphur.

Comparing the above result with those obtained by previous investigators, we note above all that ROSENHEIM and KAJIURA did not find any

Comparison of rice protein with those of other cereals.

	Carbon.	Hydrogen.	Nitrogen.	Oxygen and sulphur.
Globulin, Edestin of barley ¹	50.88	6.65	18.10	24.37
Globulin of rye. ²	51.19	7.40	18.19	23.88
Globulin, Edestin of wheat ³	51.03	6.85	18.39	23.04 0.99
Globulin of rice (No. 1 sample) ..	43.95	7.45	13.80	35.80
Globulin of rice (No. 2 sample) ..	48.95	7.70	16.20	27.15
Globulin of rice (2ndary No. 1 sample)	48.60	8.60	11.73	23.09

Zein of maize (Ritthausen)	54.69	7.51	15.58	21.53	0.69
Zein of maize (Chittenden, Osborne).	55.23	7.26	16.13	20.78	0.69
Zein of maize (Dennstedt, Harster).	54.28	7.27	16.00	21.68	0.77
Mucedin of rye ⁴	53.61	6.79	16.84	22.26	0.50
Hordein of barley ⁵	54.29	6.80	17.21	20.87	0.83
Gliadin of wheat ⁶	52.72	6.86	17.66	21.62	1.14
Gliadin of rye ⁷	52.75	6.86	17.72	21.48	1.21
Prolamin of rice (No. 1 sample)	—	—	14.44	—	—

1. ASBORNE. Jour. Americ. Chem. Soc. Vol. XVIII, No. 7, July 1895 p. 566.
2. " " " " " Vol. XVII, No. 6 June 1895 p. 438.
3. ditto.
4. RITTHAUSEN. Die Eiweiss Körper (soluble in 85 % alcohol). 1872.
5. OSBORNE. Jour. Americ. Chem. Soc. Vol. XVII, No. 7, 1895, p. 566.
6. OSBORNE and VOARHEES. Jour. Americ. Chem. Soc. Vol. XV, No. 6, 1895 p. 78.
7. OSBORNE. Jour. Americ. Chem. Soc. Vol. XVII, No. 6, 1895 p. 440-441.

	Carbon.	Hydrogen.	Nitrogen.	Oxygen and sulphur.
Prolamin of rice (No. 2 sample) ..	58.50	9.80	13.92	17.78
Prolamin of rice (2ndary No. 1 sample)	58.115	8.98	10.70	22.205
Prolamin of rice (2ndary No. 2 sample)	60.16	9.40	11.00	19.64

Glutenin family :—

Glutenin, Casein of rye, ¹	52.14	6.93	16.38	23.49 1.06
Glutenin of wheat ²	52.34	6.83	17.49	22.26 1.08
Oryzenin of rice (No. 1 sample) .. (containing some prolamin).	55.85	7.60	14.65	21.90
Oryzenin of rice (No. 2 sample) .. (containing some prolamin)	50.50	8.65	17.10	23.75
Oryzenin of rice (No. 1 sample) .. (containing no prolamin)	50.15	8.80	17.10	24.09
Oryzenin of rice (No. 2 sample) .. (containing no prolamin)	49.50	8.50	15.50	26.50
Oryzenin of rice (2ndary No. 1 sample)	56.50	8.30	15.30	19.90
Oryzenin of rice (2ndary No. 2 sample)	41.80	8.10	16.35	33.75

From this table we see that two of the samples of rice globulin and two of rice glutenin are notably different from those of other cereals, and that the nitrogen content of rice prolamin is comparatively less.

III. Is there any Difference among these Protein Matters in their nutritive value for lower Fungi?

The object of this series of experiments was to find out to what extent the above mentioned proteins could be utilized by saké yeast and *Aspergillus Oryzae*, and whether there was any difference among them in their nutritive value for these organisms.

The solutions used were as follows :—

1. Koji extract, its total nitrogen already determined.
2. Nitrogen-free HAYDUCK's solution.³

1. RITTHAUSEN. Die Eiweisskörper. 1872. p. 83.

2. OSBORNE and VOARHEES. Jour. Americ. Chem. Soc. Vol. XV, No. 6, 1895 p. 79.

3. The cane sugar used for Hayduck's solution was purified as follows :—baryta was added to the sugar solution, heated until no ammonia reaction could be observed in the distillate. The main part of the barium was precipitated with sulphuric acid and the rest with carbon dioxide gas.

To this second solution, the above mentioned proteins were added each separately until the nitrogen content became equal to that of the Koji extract. The above solutions were fractionally sterilised during four days, once every day during 40 minutes at 60-70° C.

A definite quantity of saké yeast (the yeast was grown in a solid culture medium, then transferred into nitrogen-free HAYDUCK's solution, and after mixing very well a definite quantity of the liquid was taken) and a small quantity of the spores of *Aspergillus Oryzae* were added to each flask. The flasks were then placed in a thermostat at about 25° C and after several days the amount of their multiplication was determined:—the yeast by number, the mould fungus by weight.

In the table reproduced below, each flask from (A to O) contained 80 c.c. nitrogen-free HAYDUCK's solution, 8 c.c. yeast and one platinum-ear full of the spores of *Aspergillus Oryzae* and the following quantities of the proteins:—

Flasks

A	Albumin	No. 1		10.4500 gr.
B	Albumin	No. 2		19.0100 gr.
D	Globulin	No. 2		0.3871 gr.
E	Prolamin	No. 1		0.4343 gr.
F	Prolamin	No. 2		0.4506 gr.
G	Oryzenin (containing some prolamin)	No. 1		0.4281 gr.
H	Oryzenin (do.)	No. 2		0.3662 gr.
I	Oryzenin (containing no prolamin)	No. 1		0.3560 gr.
J	Oryzenin (do.)	No. 2		0.4046 gr.
K	Globulin (secondary sample)	No. 2		0.5347 gr.
L	Prolamin (do.)	No. 1		0.5861 gr.
M	Prolamin (do.)	No. 2		0.5702 gr.
N	Oryzenin (do.)	No. 1		0.4099 gr.
O	Oryzenin (do.)	No. 2		0.3836 gr.

Koji extract 100 c.c. (0.0784 % N) + 10 c.c. yeast + one platinum-ear full of the spores of *Aspergillus Oryzae*.

HAYDUCK's solution 100 c.c. + 10 c.c. yeast + one platinum-ear full of the spores of *Aspergillus Oryzae*.

The manner of propagation of the organisms in these flasks was as follows;—

It is seen that all the proteins except prolamin were utilized by saké yeast and *Aspergillus Oryzae*, though these organisms behaved differently. The multiplication of the yeast in n (oryzenin), k (globulin), o (oryzenin), i (oryzenin), j (oryzenin) and b (albumin) approached that of the control culture in Koji extract. On the other hand, in the cultures g and h (both oryzenin) the multiplication was less, compared with that of the control, probably on account of its preparation:—Oryzenin was prepared in this case by treating with 0.2% *Na OH* solution without extracting prolamin.

As to *Aspergillus Oryzae* which developed in all the cultures except those containing prolamin, b (albumin), and d (globulin) the multiplication was greater than in the control Hayduck's solution or nearly equal; the development was particularly good in i (oryzenin)—, j (ditto)—, and k (globulin)-flasks.

With regard to the quantity of the alcohol produced, all except prolamin flask, a (albumin)—, d (globulin)—, and g (oryzenin)-flasks approached that of the control. In the a (albumin)—, d (globulin) and g (oryzenin) flasks there were found very small quantities of alcohol and none in those containing prolamin.

As to the acids formed, the less there was of them the less there was of alcohol.

Our experiments leave no doubt that in all media containing prolamin neither the yeast nor the mould fungus can thrive. It is plain that although our prolamin lacks the special properties of OSBORNE'S prolamin, yet it is quite different from albumin, globulin, and oryzenin:—proving indirectly the presence of prolamin in rice.

Conclusion.

1. About the common constituents of rice, our studies have brought nothing new to light. The further the whitening process is carried the less the quantity of fat becomes, and as a general rule the best rice contains least fat; there are, however, exceptions.

2. With respect to the quantity of protein nitrogen, we have not been

able to find out any difference between the rice of Nada, which was whitened especially well and that of other districts, in which the whitening was less complete. As it is evident from the analyses made by many investigators, that rice bran is rich in protein, so we may infer that the rice of the Nada districts should contain more protein than that of other districts, if the whitening be carried to the same degree in both.

3. As to the ash content, the relation between Nada rice and that of other districts is the same as in (2).

4. The rice examined by us contained four kinds of proteins, viz. albumin, globulin, prolamin, and oryzenin.

5. The three kinds of proteins, albumin, globulin and oryzenin, are utilized by saké yeast and *Aspergillus Oryzae*; prolamin is not.

On the Physiological Difference of the Varieties of *Aspergillus Oryzae* employed in the Three Main Industries in Japan, namely Saké-, Shōyu-, and Tamari-Manufacture.¹

BY

Teizō Takahashi and Takeharu Yamamoto.

In Japan, the fungoid mass of *Aspergillus Oryzae* ("kōji") is employed almost in all the branches of the brewing industry. The chief branches which use kōji as raw material and are important to the financial and hygienic conditions of the nation, are the "saké"-, "shōyu"- and "tamari"-industries, so it is only natural that we should pay special attention to the nature and the properties of the fungus. One of the writers² has already reported on the occurrence of the three varieties (α , β , γ) of *Aspergillus Oryzae*, which are chiefly used for the preparation of saké. If we accept the above fact on *Aspergillus Oryzae*, it can not be questioned that the fungus employed in the saké industry differs in properties from the other varieties employed in the other branches of industry, especially when we consider the differences between the raw materials employed in the different branches of the brewing industry. The chief raw material of saké manufacture is rice, which is rich in starch and poor in protein; whereas in the case of "shōyu", the main raw materials are soy-beans, predominating in protein and almost absent of starch, and barley; while in "tamari" soy-beans are chiefly used. If we can apply the theory of use and disuse, the varieties accustomed to grow chiefly on rice will acquire, by and by, the

1. This report has already been published in the Jozōshikenjo Hōkoku (the Report of the Brewing Institute at Tokyo), March, 1911.

2. Jour. of the Coll. of Agric., Tokyo Imp. Univ., Vol. I. No. 1.

property of increasing the secretion of the diastatic enzyme, and decreasing the peptonifying ones, and vice versa in the case of that grown constantly on soy-beans.

I. The Varieties used in the Experiments.

The varieties used in the experiments are 16 in number, among them three varieties are described already in the former report¹, so the other thirteen varieties will be explained below.

Variety.	Remarks.
	In koji-extract agar surface culture, (at 25°C, 4 days.)
Variety A	Conidiophore long, with few spores of light brownish yellow color. Isolated from "tane ² -koji" of saké manufacture.
Variety E	Isolated from "tane-koji" of saké manufacture. Rich in brownish yellow spores. A white, light slender mycelium entangles between the conidiophores, like the web of a spider.
Variety F	Isolated from shōyu-koji at a factory of Chōshi city. Very rich in spores, which are brownish yellow in color.
Variety G	Isolated from the same sample as variety F. Rich in spores but it has fewer conidiophores. The color of the spores is dark reddish brown, especially in the margin part of the colony.
Variety H	Isolated from shōyu-koji at Chōshi city, but from a different sample than the F and G varieties. Very poor in spores, which are colored from yellowish green to dark yellowish green. The conidiophore is <i>very long</i> when compared to the other varieties.
Variety I	Isolated from shōyu-koji at a factory of Noda city. The external appearance of the growth is very similar to variety F.

1. Jour. of Coll. of Agric. Tokyo Imp. Univ. Vol. I. No. 1.

2. "Tane" means a seed, so "tane-koji" is a spore of *Aspergillus Oryzae*, especially prepared for this purpose.

Variety J	Isolated from shōyu-koji at a factory of Noda city, but from a different sample than variety I. The growth is rich in dark greenish yellow spores. Conidiophore develops very thick, but its length is shorter than that of variety H.
Variety K	Isolated from tamari-koji at a factory of Handa city. Rich in spores, which are strongly colored a <i>brownish yellow</i> . Conidiophores develop very thickly. It has a very similar appearance to variety G.
Variety L	Isolated from the same sample, from which variety K was obtained. Rich in <i>dark green</i> colored spores. Conidiophore is very thickly developed on the nutriment.
Variety M	Isolated from tamari-koji from Mie prefecture. The appearance of the growth is very similar to that of variety H.
Variety N	Isolated from the same sample from which variety M was obtained. The appearance of the growth is very similar to that of variety F.
Variety O	Isolated from tamari-koji from Mie prefecture. Rich in spores, but the development of conidiophores is thin. The spores are colored dark brown, dense in the periphery of the colony.
Variety P	Isolated from shōyū-koji at a factory of Noda city. Rich in yellowish green spores. Conidiophore is short.

II. The Preparation of Koji and the Method of Analysis.

The above mentioned pure fungus of each variety was infected to the boiled and sterilised whitened rice (100 g.) contained in ERLÉNMEYER'S flask, and held in a thermo-regulator during 33·30 hours. The temperature of the apparatus is shown in the following table.

The first preparation of Koji.

Days.	Time.	Temp. of apparatus.	Remarks.
First.	5. P.M.	28° C	
Second.	9. A.M.	26° C	
„	Noon.	21° C	
„	2.30 P.M.	32.5° C	Shaking and mixing the materials.
„	5.30 „	33° C	„
„	10.30 „	33.5° C	„
Third.	6.30 A.M.	37° C	O and G varieties commenced to form spores.
„	10.30 „	36.5° C	
„	2.30 P.M.	35° C	

The second preparation of Koji.

Days.	Time.	Temp. of apparatus.	Remarks.
First.	4. P.M.	30° C	
Second.	8. A.M.	30.5° C	
„	Noon.	31° C	Shaking and mixing the materials.
„	4. P.M.	32° C	„
„	8. „	34.5° C	„
Third.	5. A.M.	38° C	
„	10. „	38.5° C	G and K varieties commenced to form spores.
„	4. P.M.	37° C	

In the third preparation of Koji the change of the temperature of the apparatus was almost the same as that in the second case.

The determination of the amount of sugar, organic acids, esters, and amino-acids¹ was made with the dilute alcoholic (45%) extracts of Koji, with a special precaution to destroy the enzymic activity at the beginning of the extraction. For this purpose, koji was introduced into a flask connected with a reverted cooler and containing 200 c.c. of 90% alcohol, the material was boiled for 20 minutes.

The determination of ammonia was made with a special sample i.e. the whole mass of koji grain obtained from 100 g. of rice was taken and subjected to WURSTER's method.²

1. SÄRENSEN's formol method.

2. *Centbl. f. Physiol.* 1, 485 (1888).

III. The Content of Sugar in the whole Mass of the prepared Koji, namely the Growth on 100 g. of rice.

Varieties.	In the Experiment.			Sum of the three cases.	Order in regard to the quantity of sugar.	The fungus Percent-age in the sample.
	I.	II.	III.			
From saké tane -koji.	<i>α.</i>	7.813 g.	15.994 g.	6.060 g.	29.868 g.	III. 90%
	<i>β.</i>	6.640 „	13.761 „	4.980 „	25.381 „	V. 50%
	<i>γ.</i>	7.946 „	16.175 „	6.060 „	20.182 „	II. 83%
	<i>A.</i>	9.413 g.	18.704 g.	6.160 g.	34.277 g.	I. 100%
	<i>E.</i>	6.906 „	15.055 „	3.700 „	25.762 „	IV. 100%
From shōyu -koji.	<i>F.</i>	4.933 g.	11.737 g.	4.280 g.	20.950 g.	X. 75%
	<i>G.</i>	0.413 „	5.762 „	0.344 „	6.519 „	XIII. 25%
	<i>H.</i>	6.187 „	8.056 „	5.580 „	19.822 „	XI. 26%
	<i>I.</i>	5.573 „	13.662 „	4.680 „	23.916 „	VIII. 100%
	<i>J.</i>	5.440 „	12.932 „	4.510 „	22.882 „	IX. 100%
	<i>P.</i>	4.000 „	10.329 „	3.000 „	17.329 „	XII. —
From tamari -koji	<i>K.</i>	0.533 g.	1.652 g.	0.064 g.	2.249 g.	XIV. 45%
	<i>L.</i>	9.013 „	—	7.160 „	—	I or II. 8%
	<i>M.</i>	6.907 „	11.560 „	5.680 „	24.147 „	VII. 20%
	<i>N.</i>	6.107 „	13.615 „	5.380 „	25.102 „	VI. 67%
	<i>O.</i>	3.200 „	3.713 „	—	—	XIII or XIV. 100%

Spore formation after further 14 days at 20° C.

G. 15.120 g.
K. 12.920 „

From the above table, we can conclude that the formation of sugar is most energetic in the varieties of saké-koji and weakest in tamarikoji, with the exception of *L* variety. While the variety *I* is found very rarely (8%) in comparison to the whole mass of the original sample, this variety plays a very small rôle in the ripening of tamari or is perhaps almost indifferent in it

IV. The Amounts of Organic Acids.¹

Varieties.	I.	II.	III.
$\left\{ \begin{array}{l} \alpha. \\ \beta. \\ \gamma. \end{array} \right.$	3.2 2.7 3.3	5.6 4.6 6.8	3.2 2.8 2.6
$\left\{ \begin{array}{l} A. \\ E. \end{array} \right.$	3.8 4.3	5.0 6.0	3.0 4.6
$\left\{ \begin{array}{l} F. \\ G. \\ H. \\ I. \\ J. \\ P. \end{array} \right.$	3.5 4.9 3.8 4.4 3.8 2.9	5.0 6.2 5.6 5.2 5.6 6.2	3.0 5.0 — 3.2 — 3.6
$\left\{ \begin{array}{l} K. \\ L. \\ M. \\ N. \\ O. \end{array} \right.$	4.4 3.1 — — 4.3	5.2 4.0 4.6 6.6 6.2	3.2 2.8 3.2 3.0 —

Thus, the amounts of acids formed are almost equal in all the varieties.

V. The Esters.

The estimation of the esters was conducted with alcoholic extract, so perhaps, a small part of them escaped the estimation, but generally saké koji gave a higher percentage than tamari or shōyu koji.

For the purpose of examination of the character of the esters, fresh koji was treated by a current of steam and the distillate therefrom was saponified by alkali. The distillate, obtained when the saponification was

1. The amounts of the acids are shown in the table c.c. m. of 1/10 N. solution of natron lye, which neutralizes the acids formed by the fungus, grown on 100 g. of rice.

finished, contained amyl-alcohol¹ and other higher alcohols. The nature of the acids will be reported hereafter, but the fact that the flavour of koji is the ester of some higher alcohols is worthy of remark.

VI. The Amino-acids and Ammonia.

Varieties.	In the experiment.			Sum.	The quantity of ammonia.
	I.	II.	III.		
α .	0.078	0.079	0.117	0.274	—
β .	0.069	0.127	0.098	0.294	0.0957 g.
γ .	0.089	0.108	0.107	0.304	0.0887 „
A .	0.118	0.127	0.098	0.343	— „
E .	0.098	0.167	0.117	0.382	0.0546 „
F .	0.098	0.138	0.108	0.344	0.0990 „
G .	0.089 (^{spore state} 0.207)	0.098	0.198	0.285	0.0580 „
H .	0.108	0.138	0.137	0.383	0.0767 „
I .	0.079	0.167	0.098	0.344	0.0956 „
J .	0.079	0.167	0.098	0.344	—
P .	0.108	0.167	0.117	0.392	0.0580 „
K .	0.050	0.098	0.078	0.226	0.1160 „
L .	0.089	—	0.148	—	0.0680 „
M .	0.148	0.098	0.067	0.313	0.1024 „
N .	0.098	0.098	—	—	—
O .	0.069 (^{spore state} 0.222)	0.127	—	—	0.0580 „

Thus the amounts of amino-acids formed alter, when the temperature of the growth is changed, even with the same variety. Perhaps the difficulties of an even growth of the fungus will be another cause of the disturbance of the parallelism in the quantities of amino-acids formed. But generally, the varieties obtained from shōyu koji or tamari koji have the tendency to form a rather greater quantity of amino-acids or ammonia than that of saké koji. The exception to this general rule is the case of *K*

1. Amyl-alcohol was tested with anis-aldehyd and conc. sulphuric acid.

variety, which was isolated from tamari koji, generating only 0.226 g. of amino-acids from 100 g. of rice, but if we observe the amounts of ammonia formed, the fact will be recognized very easily by reason of the large quantity of the latter. The amounts of ammonia and amino-acids are relatively small in the case of *G* variety, though it was isolated from shōyu koji. The reason why such phenomenon occurs must be a subject for further investigation.

Moreover, the increase in the amount of amino-acids, when the fungus grows very old with dense colored spores, is shown in the case of the varieties *G* and *O*, and this fact coincides with the former observation¹ that the liquefaction of the gelatine commences almost always in the old culture which developed many spores.

V. The Liquefaction of Gelatine in the Culture of the Fungus in Koji Gelatine.

The fungus was infected to the inclined surface of koji gelatine (10 c.c.), which was introduced into the test tube with the same diameter kept at 15—20° C and after 45 days the volume of gelatine which liquefied was compared.

Varieties.		Degree of liquefaction ²		Sum.	Order of liquefaction from weakest to strongest
		I.	II.		
From tane- koji of saké.	<i>α.</i>	2.	3.	5.	3.
	<i>β.</i>	6.	7.	13.	6.
	<i>γ.</i>	3.	1.	4.	2.
	<i>A.</i>	1.	2.	3.	1.
	<i>E.</i>	4.	6.	10.	4.
From shōyu koji.	<i>F.</i>	11.	9.	20.	9.
	<i>G.</i>	14.	14.	28.	12.
	<i>H.</i>	9.	8.	17.	8.
	<i>I.</i>	5.	10.	15.	7.
	<i>J.</i>	12.	12.	24.	11.
	<i>P.</i>	10.	11.	21.	12.

1. Jour. of College of Agric. Tokyo Imp. Univ. Vol. I. No. 1.

2. The smallest degree of liquefaction is exhibited by 1 and so on.

From tamari koji.	<i>K.</i>	13.	15.	28.	12.
	<i>L.</i>	11.	9.	20.	9.
	<i>M.</i>	7.	4.	11.	5.
	<i>N.</i>	8.	5.	13.	6.
	<i>O.</i>	15.	13.	28.	12.

The degree of the liquefaction of gelatine is weaker in the culture of saké koji than in that of shōyu or tamari koji. Nevertheless, in comparing shōyu koji to tamari koji, we anticipated an energetic liquefaction of gelatine in the latter, but *M* and *N* varieties did not agree with this anticipation, giving weaker action in comparison to shōyu koji culture.

Summary.

The physiological differences between the fungus of saké koji, shōyu koji and tamari koji are remarkable, especially in the formation of amino-acids, sugars, esters, ammonia and the liquefaction of gelatine. There are differences, as formerly reported, between the varieties of *Asp. Oryzae* used in the saké industry, but the differences between the varieties of the fungus isolated from the three different industries are more remarkable than the differences existing between the varieties which belong to the same branch of industry.

Above all, the assumption made in the beginning, may be repeated here, but now as a definite proposition:—the formation of sugars is more conspicuous in the varieties of the fungus customarily used in the brewing of saké than in that of the varieties of shōyu or tamari, and vice versâ in regard to the peptonification on the formation of amino-acids or ammonification.

Still further, the formation of large quantities of amino acids at the stage of the spore formation of the fungus, inspite of their being less of them at the mycelium stage, explains why we use koji at spore stage in shōyu or tamari industry, and at the mycelium stage in saké brewing.

On the Natural Gigantic Colonies of Yeast.

BY

Teizō Takahashi.

With Plate VIII.

The observation of gigantic colonies of yeast, offered by LINDNER, is one of the most convenient methods for the identification of the varieties of yeast, but the colony formed from a single cell is more natural. In the common plate culture we could find scarcely any difference between the forms of the colonies of the yeast varieties, although they are quite different in their other physiological properties, while some small precautions in the method of the culture will show us the proper form of the colony according to the differences of yeast varieties.

The gigantic colony formed in the plate culture will be called here "*natural gigantic colony*," to distinguish this from LINDNER's gigantic colony which is formed from the drop of the yeast culture.

The formation of the natural gigantic colony is observable, if we dilute as much as possible the common gelatine plate culture to the extent of that in the third plate of PETRI's dish, we can find only two or three colonies, which have been kept a comparatively long time. The duration of the culture varies according to the temperature of the room, but generally 30-50 days are sufficient for this purpose.

In the plate, there are represented some varieties of *Saccharomyces saké*, Yabe, and one of the varieties of beer yeast.

The colony of B 33 is one of the peculiar colonies of saké yeast. The surface is almost smooth except the terrace-like elevations, and very fine concentric rings on the margin part. The varieties A 6, A 22, A 27, A 31, A 29, B 12, B 18, B 24, B 30, are similar in their properties having

radiated wrinkles on the surface of the margin part of the colonies. The radiations are most evident and long in *A* 31 and least so in *A* 6. In *B* 12 and *B* 30, the radiations are very short. The varieties *A* 6, *A* 31, *A* 27, *A* 22, *B* 12, *B* 18, *B* 30 have concentric rings on the surface of their colonies and in some of them (*A* 31, *B* 18) have the terraced appearance, while in the other varieties (*A* 29, *B* 24) they are destitute of these concentric rings or the terrace-like appearance and have a round concavity in the central part.

The form of the colony of variety *B* 19 is very peculiar in comparison to the other varieties of saké yeast and has a rather close similarity to the Bulton beer yeast. The wrinkles on the margin part in both cases are irregular with a feathery appearance and they are separated by deep furrows.

The varieties *A* 19, *A* 32, *B* 25, *B* 9, *B* 27, have similar appearances with fan-like compact streams on their marginal part. They form some terrace-like appearances (*A* 32, *B* 25, *B* 9, *B* 27), with the exception of variety *A* 19. Variety *B* 9, has a special appearance with a furrow-like concavity around the central elevation. In this regard, variety *B* 5 has the same furrow, but it has radiated wrinkles instead of compact fan-like wrinkles on the margin.

Variety *A* 13 has an almost similar appearance with variety *A* 27, but in the former case the radiated lineations and wrinkles are seen over the whole surface, while in the latter case the wrinkles are limited to the marginal part, and they are compact and broad.

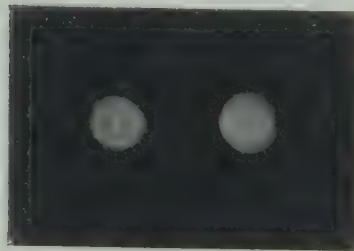
A very peculiar colony is observable in the case of variety *B* 2. The wrinkles on the marginal part have a close similarity to Bulton yeast in a certain degree, but differing in their compactness and non-divisibility of the marginal part.

The conclusion from the above data is as follows :

The gigantic colonies formed by the single cell are very characteristic in their forms and appearances according to their different varieties. The method of their formation is very convenient and more natural than LINDNER'S gigantic colony for the identification of the varieties of the yeast.

EXPLANATION OF PLATE VIII.

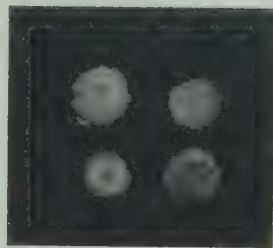
Natural gigantic colonies of several varieties of saké yeast, *Saccharomyces* saké and Bulton bear yeast (in koji extract gelatine). (Details in the text.)



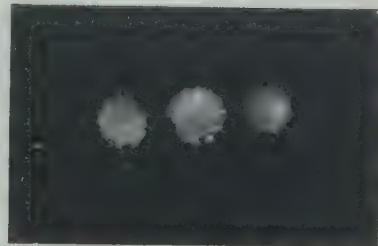
A. 6.
(10/IV-9/V)



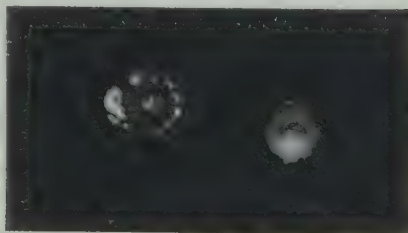
A. 31.
(7/IV-28/IV)



B. 24.
(17/III-16/IV)



A. 27.
(7/IV-2/VI)



A. 19.
(4/IV-9/V)



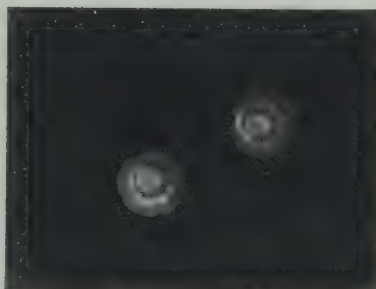
B. 2.
(2/XII-20/II)



A. 22.
(24/III-27/IV)



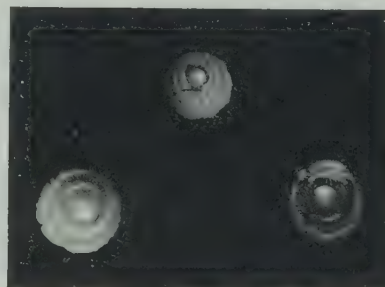
B. 19.
(5/V-2/VI)



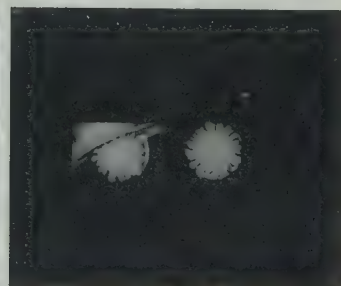
A. 32.
(7I/V-19/V)



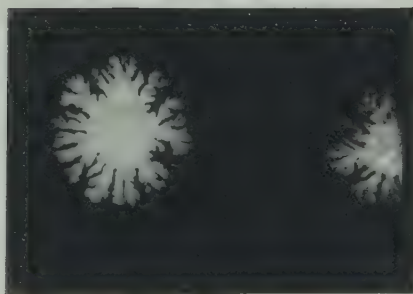
B. 12.
(15/IV-16/V)



B. 25.
(10/IV-16/V)



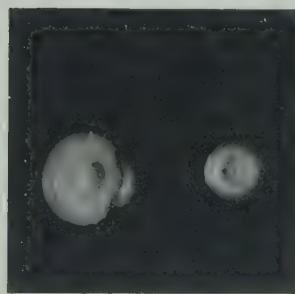
A. 29.
(12/V-2/VI)



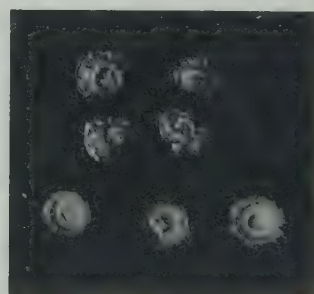
Bulton.
(27/IV-2/VI)



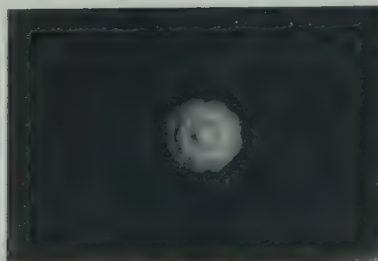
B. 18.
(7/III-16/IV)



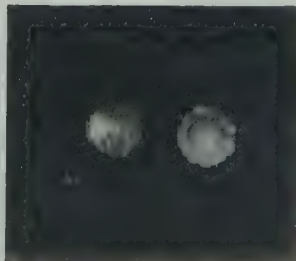
B. 33.
(17/III-30/IV)



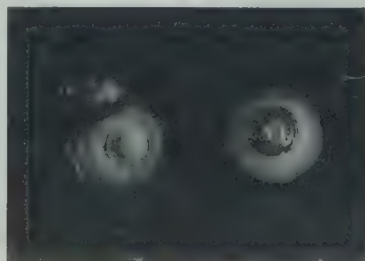
B. 5.
(10/XII-20/II)



B. 9.
(7/III-30/IV)



A. 13.
(24/III-28/IV)



B. 27.
(10/III-30/IV)



B. 30.
(7/IV-19/V)

On the Detection of Amylalcohol and other Aliphatic Alcohols and their Esters.

BY

Teizō Takahashi.

On the detection and determination of fusel oil, the writer's method, a modification of KOMOROWSKI's, has already been reported,¹ but there are some difficulties when we apply the method, in the case of the determination, to the distillate of saké; though it may be used safely to the distillate of the culture of the yeast in koji-mash or other fluidal nutriments. Nevertheless, the improvement of the method was neglected by the writer, while recently TAKEO KOSHINO² has reported on his researches on one of the writer's processes, the vanilline sulphuric acid process, and pointed out the author's mistakes in the observation of the color formed when the testing fluid was poured on the mixture of vanilline and concentrated sulphuric acid. His assertion that the true color formed, when the mixture contains fusel oil, is purplish red and not blue³ is right. He altered, therefore, the writer's method as follows.

“Take 0.5–1.0 cc of the testing fluid in the test tube and add 2 cc of concentrated sulphuric acid, and after well shaking add 0.1 cc of 10% solution of vanilline in absolute alcohol and afterwards 1 cc of water. Compare the color developed after well shaking with the control test, which contains a definite quantity of fusel oil in the alcoholic fluid, of

1. T. TAKAHASHI. Bulletin of the College of Agric. Tokyo, Imp. Univ. Japan, Vol. VI, No. 1, p. 437.

2. TAKEO KOSHINO. Journal of the Pharmaceutical Society of Japan, No. 351.

3. In the writers's former report, it was described as blue.

which the percentage of alcohol is as near as possible to that of the testing fluid."

His affirmation that the blue color develops by the mere presence of ethylalcohol in the test is worthy of my gratitude but, in spite of finding ethylalcohol a hindrance, he still uses vanilline in *alcoholic solution* in the determination of fusel oil. From this standpoint, the writer proceeds to exclude ethylalcohol, with the exception of that which is naturally found in the testing fluid.

For this purpose the writer dissolved 1 gram of vanilline in 200 cc of sulphuric acid with a specific gravity of 1.84. The vanilline solution thus prepared was introduced into the burette, from which 2 cc was taken in the test tube, and adding 1 cc of the testing fluid, the water was poured in drop by drop and the mass shaken at each additional drop. The proportion of vanilline and sulphuric acid to the testing fluid is quite the same as that in KOSHINO's process.

The comparison of the delicacy of the test was made with 0.002% fusel oil in 15% alcohol.

KOSHINO's process.

A slight dark shade occurred after addition of 20 drops of water.

After a further addition of 1 cc of water a trace of red color was observable, when the fluid was looked at from above, while this was not the case if seen from the side of the tube.

The writer's process.

A heavy dark shade occurred after addition of 20 drops of water.

Further addition of 1 cc of water produced a *purplish red* coloration, which could even be observed from the side of the test tube.

Thus, the modified process may be applied safely in very diluted solutions of fusel oil such as 2:100000. The cause of the modification by the addition of drops of water consists in the fact that the coloration which occurs in the presence of amylalcohol, depends largely on the concentration of sulphuric acid. So that the characteristic coloration formed by the test may disappear altogether when we dilute the reaction product with large

excess of water. Moreover the coloration will reappear at once, if the concentration of the acids is increased.

The change of the shade in coloration will be perceived more clearly by the following test.

If to 0.5 cc of 0.1% amylalcohol¹ in 15% pure ethylalcohol, 2 cc of the above mentioned vanilline sulphuric acid solution is added, there appears instantly a *red* coloration changing gradually to *intense red*, then becoming dark. While the addition of pure water drop by drop decreases the darkness in accordance to the quantity of water added. A further addition of 10 drops of water in a stratum, shortly afterwards changed to a purple color, which increased in intensity on shaking and after the addition of a still further 3 cc of water. The purple colored layer altered to *bluish purple* by the further addition of 1 cc of water, but the bluish purple layer altered to a deposit of the same color after 10 days standing, leaving a *rose red colored* clear layer upon it.

The coloration brought into view by many other alcohols, is tabulated below:—The test was made in the test tube, and the water was added drop by drop from the burette, using 2 cc of vanilline sulphuric acid solution for each determination.

Alcohols.	Remarks.
Ethylalcohol. (15%) (20drops)	No coloration perceived on the addition of water.
Absolute ethylalcohol (1 cc)	A yellow coloration was perceived when the reagent was mixed with the testing alcohol. The coloration decreased after the addition of 9 drops of water, while the addition of 15 drops caused a dark coloration which remained unaltered up to 21 drops of water. Still further addition of 1 cc of water caused a slightly <i>dark greenish blue</i> coloration, which changed into <i>light green</i> color when the water added amounted to 2 cc.

1. Manufactured by MERCK's factory in Germany. It was colorless, but contained a trace of furfurol,

Methylalcohol. (one drop).	There was no change even after the addition of 20 drops of water, but <i>dark red color</i> was observable when the amount of water attained to 30 drops. A slight <i>purple</i> tinge was added when the amount of water added increased to 50-60 drops and the intensity of the coloration was quite the same even after another addition of 1 cc of water. After 12 days there was found a dark colored deposit, over which the fluid remained clear with light dark and reddish yellow coloration.
Methylalcohol. (large drop).	A <i>reddish yellow</i> coloration was perceived after the addition of only 15 drops of water increasing in intensity by shaking.
Propylalcohol. (with boiling point of 96-99° C) (one drop)	An intense yellow coloration was perceived after the addition of 8 drops of water, while the colored layer changed to an intense <i>purplish red</i> when the water (7 drops) added was laid a while in a stratum without shaking. The color changed to <i>intense red</i> by shaking, thence the fluid was constantly shaken, the water added drop by drop, and the whole fluid changed to purple when the amounts of water added reached 2 cc. Further addition of 4 cc of water changed the color to <i>purplish-blue</i>. After 10 days an indigo colored flocculum floated on the surface of the fluid, leaving a transparent but light purple rose colored fluid underneath it.
Iso-propyl- alcohol. (one drop).	An intense yellow color was perceivable after the addition of 8 drops of water, as in the case of normal propylalcohol. The further addition of 7 drops of water without shaking, and left a while in a stratum, gave <i>purple red</i> color to the layer of water. An <i>intense red</i> color appeared in the whole fluid after shaking. Thence, adding the water drop by drop and shaking at every

addition, an intense *purple* color was perceived when the amount of the water reached 3 cc (after 20-30 minutes). Further addition of 3 cc water gave to the coloration a somewhat reddish tinge, while the intensity of the color decreased when the whole amount of water was brought up to 8 cc. After 10 days a dirty and slightly purplish colored flocculum floated over the surface of the fluid, transparent and light yellow.

From the above mentioned results *normal propylalcohol and iso-propylalcohol may be distinguished by their coloration*; because the coloration perceived at the end of the test is from *red to purplish red* in the latter case, and *purplish red* in the former.

Alcohols.	Remarks.
n-Butylalcohol. (one drop).	An intense <i>yellow</i> color was perceived after the addition of 5 drops of water. The color changed to red after the addition of 10 drops, the intensity increased after the addition of 20 drops of water. If 10 drops of water were laid over this in a stratum, they changed to <i>purple</i> color. The whole fluid became a dark purple color after shaking, while the red color reappeared, accompanied by the original purple colors on the addition of water amounting to 9 cc. The intensity of the <i>red coloration</i> gradually increased with the further addition of water, but a slight trace of <i>purple tinge</i> remained even after the increase reached 14 cc. After this operation a part of the fluid was taken in another test tube, and water from 5 to 6 times its volume was poured in, but there was <i>no</i> occurrence of the <i>blue tinge</i> . 10 days afterward a brown colored deposit was perceived, over which a rosy, reddish yellow colored fluid remained clear.

Amylalcohol. (Merck pure). (one drop).	An <i>intense yellow coloration</i> is perceivable after the addition of 4–8 drops of water. The color changed to <i>red</i>, when the amount of the water attained to 15 drops. Hereafter, 5 drops of water were laid in a stratum over the colored fluid, after a while a <i>reddish purple</i> coloration in the layer of water was perceived. After this, the fluid was shaken constantly and the water was added continuously drop by drop until the amount of water attained to 45 drops. By this time the <i>purple color</i> increased suddenly and changed to opaque. By further increase of the water from 1–3–6 cc the <i>red coloration</i> reappeared.
Esters.	Remarks.
Amylacetate. (one drop).	An <i>intense red</i> coloration occurred after the addition of 4 drops of water. Further 5 drops of water laid over it in a stratum after a while obtained an <i>intense purple</i> coloration, which increased in intensity on shaking and changed to <i>purplish rose red color</i>. Further 10–16 drops of water laid in a stratum gradually assumed a <i>purplish</i> tinge with an opaque ring at the contact surface of the stratum. The whole fluid became non-transparent after shaking, increasing its intensity until the amount of water added reached 3 cc, while at 4 cc the opacity decreased in a certain degree and some 20–30 minutes afterwards the color changed to <i>red</i>, namely to <i>cinnabar red</i>. After standing overnight the part in which the red coloration was perceived changed to a clear fluid lying over an oily matter of a dense <i>crimson red</i> coloration with purple shade.
Amylformate. (one drop).	A <i>clear yellow</i> color appeared after the addition of 4 drops of water and there was no change in the color until

	the amount of water attained to 10 drops. The color changed to red, when the amount of water increased to 15 drops. After this, 5 drops laid over the fluid in a stratum after a while obtained a <i>purple</i> tint. Thence the fluid was constantly shaken while adding water drop by drop, and when the amount of the latter attained to 2 cc, there appeared a <i>purplish red</i> coloration of an opaque aspect. The opacity increased when 4 cc of water was added, but commenced to decrease at 5 cc. At 8 cc of water, a <i>purple tint</i> still remained, but at 10 cc a <i>red color</i> reappeared.
Amylbenzoate. (one drop).	A <i>yellow color</i> was perceived after the addition of 8 drops of water. The color changed to <i>red</i> by 15 drops and shaking. Further 5 drops laid in a stratum after a while obtained a <i>purplish red</i> tint and the <i>purple coloration</i> increased on shaking with the appearance of opacity. Still another addition of 2 cc of water changed the fluid after shaking to purple accompanied by a trace of <i>red</i>. Another addition of 1 cc caused the decrease of the coloration.
Butylether. (one drop).	When the quantity of water added drop by drop attained to 25-30 drops, there appeared a trace of a <i>green tint</i>, after which 5 drops of water laid in stratum changed the color after a while to a somewhat <i>rosy red</i>.
Amylether. (one drop).	The <i>red color</i> occurred at every addition of the water drop, but it changed to yellow on shaking. The phenomenon continued until the amount of water attained to 15 drops. Thenceforth 5 drops of water were laid in a stratum, which obtained after a while a light <i>purple red</i> coloration. The color of the fluid changed <i>thoroughly red</i> by shaking. A further addition of 1 cc of water gave the fluid an opacity, which increased with 2 cc of water. The <i>red color</i> remained, but only in a slight degree after the

	amount of water reached 3 cc, and the main coloration was replaced by <i>purple</i> , which remained the same at 4 cc of water.
Butyl- iso-valerianate. (one drop).	An <i>intense red</i> color appeared on the addition of 4 drops of water. The color altered to <i>reddish yellow</i> on shaking, and after the addition of 8 drops of water it changed to <i>crimson red</i> . After this, 15 drops of water were laid over in a stratum which obtained a <i>purple</i> tint, accompanied by a <i>red tint</i> on shaking. After this the increase of the water enhanced the intensity of the color till 2 cc of water was reached. A further addition of 2 cc of water caused the <i>purple tint</i> to change to a <i>cinnabar red</i> . An increase of water decreased the intensity of the coloration with from 3 to 6 cc successively and it became very faint at 9 cc. After standing overnight an oily matter floated on the surface of the fluid, leaving a <i>purplish red</i> colored fluid underneath it.
Propyl- valerianate. (one drop).	A clear yellow color appeared after the addition of 4 drops of water. The color changed to <i>light red</i> at 8 drops, and to <i>crimson red</i> at 15 drops. After this, 5 drops of water laid over in a stratum, after a while obtained a <i>bluish purple</i> color. With the further addition of water from 1 to 4 cc the <i>purple</i> color became predominant and opaque, while at 6 cc the intensity decreased, there was no shade of red coloration, but a change to purple altogether. After standing overnight a bluish oily matter floated on the surface of the fluid.
Acetic ester. (one drop).	Four drops of water were laid a while in a stratum over the fluid, there appeared <i>dark</i> and <i>light red</i> coloration in the stratum. The color changed to <i>dark yellow</i> after shaking. After continued shaking and adding of water drop by drop the whole fluid changed to <i>dark</i> and <i>light rosy red</i>

	when the water amounted to 15 drops. The darkness increased till the amount attained to 35 drops, while the further addition of water from 1 to 3 cc commenced to decrease it, and at 5 cc there was almost no tint of the red with a predominance of light dark yellow coloration.
Methyl- iso-butyrate. (one drop).	The red coloration began to appear after the addition of 25 to 30 drops of water. 1 cc of water laid over the fluid in a stratum for a while gained a <i>light red color</i> .
Methyl- propionate. (one drop).	A yellow color commenced to appear after 15 drops of water added. After the addition of 20 drops of water, trace of the dark shade began to appear, and when the water was increased to 25 drops, a trace of <i>red</i> color was perceived. The further addition of 1 cc water changed the color to <i>purplish red</i> and at 3 cc it became altogether <i>purple</i> .
Methyl-lactate. (one drop).	There was no change in the color of the fluid even after the addition of 15 drops of water, while at 25 drops from <i>green</i> to <i>dark green</i> color became evident. Now the <i>red</i> coloration commenced to appear and increased succeedingly on the addition of water and added to this a blue color at 4 cc. After standing overnight, a dark blue flocculum floated over the surface of the fluid with light dark and red color.
Methylacetate. (one drop).	There was no change in the fluid even after the addition of 40 drops of water, but after standing 10 days there appeared a light <i>purplish rosy red</i> coloration.
Methylformate. (one drop).	There was no change in the fluid as to its color even after the addition of 30 drops of water, while a further addition of 1 cc of water caused a light red coloration to appear and its intensity became more conspicuous with a dark shade when the added water amounted to 2 cc.

Propyonic ester. (one drop).	There was no change of coloration in the fluid even after the addition of 30 drops of water, while the layer of 1 cc of water poured over it obtained a <i>light red</i> coloration after a while.
Capronic ester (one drop).	A <i>yellow coloration</i> commenced to appear after the addition of 10 drops of water, and it changed to <i>crimson red</i> after adding 20 drops. The further addition of water laid over the fluid in stratum, gave only a trace of the purple coloration to the layer. When the water added amounted to 7 cc the grade of the color decreased and the uppermost and faintest colored part was very similar in shade to that of propyl-valerianate.
Acetacetic ester. (one drop).	A green color was observable in the fluid after the addition of 4-5 drops of water. The color changed to <i>reddish-green</i> after a further addition of one drop of water. A further addition of 2 drops and shaking changed the fluid to red which altered to <i>dark green</i> , and after thirty minutes the dark shade decreased, giving a <i>blue deposit</i> . ¹

From the above described observations, it will very easily be seen that we can detect or determine fusel oil by means of *vanilline* and *sulphuric acid*, assuming that the quantities of ethylalcohol admixed in the testing fluid is not too much. Generally, the higher alcohols produce *red*, *reddish purple* or in some cases *bluish purple* colorations by these reagents, while ethylalcohol gives blue or greenish coloration in moderate concentration. (one drop of abs. alcohol : 1.5-2 cc water.)

Methylalcohol, though simplest in its constitution, produces a red coloration by the same reagents.

1. The same test was tried with acetone, which gave a yellow color before the addition of the water. The intensity of the color increased by adding from 1 to 4 drops. At 10 drops addition the color changed to *red*, which altered to dark and opaque red after a further addition of 1 cc of water. At 7 cc a dark flocculent deposit separated in the *blue* fluid.

According to the writer's modified process, fusel oil may be detected in the dilution of 2 : 100,000.

Furfural produces a dark coloration by these reagents, and as this substance is almost a constant impurity of fusel oil, the advice given by T. KOSHINO, that the test must be applied after the removal of aldehyde in the testing fluid, must be accepted here.

The esters and ethers too behave very similarly to the alcohols from which they are derived and this is one of the defects of this test, inasmuch as all alcoholic beverages generally contain fusel oil besides their esters, but practically in the determination of fusel oil such defects may be neglected quite safely.

Anyhow, the coloration observed in the case of acetacetic ester [$CH_3-CO-CH_2-CO_2 C_2 H_5$] is characteristic, green—reddish green—red—green—blue, and may be used to distinguish this substance from acetic ester [$CH_3-CO_2 C_2 H_5$] and many other esters derived from aliphatic alcohols.

The three sorts of the methylesters, namely lactate, acetate and formate may easily be distinguished by this test, especially so in the case of lactate, which gives a green, dark green, reddish green, reddish blue green, according to the amount of the water added.

The coloration perceived when the test is applied to *acetone* may be quoted here as addenda, because, the color formed with the substance is quite characteristic, giving *red*, *dark red*, and *blue* according to the amounts of the water added.

On the Chemistry of "Mirin" and its Troubles.

BY

Teizō Takahashi, Y. Shimazu
and S. Hagiwara.

"Mirin" is a sweet liquor very common in Japan, of a yellow or yellowish brown colour and of the consistency of oil. It contains as much or more alcohol than "Saké," and has an aroma characteristic to itself. Great quantities of "Mirin," under the name of "Tosō-shu" or "Tosō¹," are drunk in every house on the occasion of the congratulations at New Year, not only by every member of the family, from the youngest to the oldest, but also when the mutual New Year's calls are made. On "Hinamatsuri" or "Sangwatsu-no-sekku," the festival of dolls in March, a kind of liquor made from "Mirin" called "Shiro-saké," a white alcoholic beverage, is placed before the dolls. But it is mainly used in cookery i.e. for the preparation of Japanese soup, or diluted "Soy" or "Shōyu" and boiled fish or vegetables to give them a better taste than boiled simply with "Soy" or "Shoyu." Its manufacture is usually connected with that of "Saké." A city celebrated for the manufacture of "Mirin" is Nagareyama, on the Yedogawa, about twenty-three miles north of Tokyo. The annual production of "Mirin" in Japan amounts to about 30,000–50,000 *koku* or 1,600,000–2,000,000 gallons in round number.

For the preparation of "Mirin," steamed "Mochi-gome," or glutinous

1. "Toso" is prepared from "Mirin" by adding the mixture of many kinds of spices i.e. *Zanthoxylum shinnifolium*, *Siler divaricatum*, *Paticodon grandiflorus*, *Cinnamomum Loureirii*, *Atractylis ovata*.

rice, “Kōji,” and “Shōchû”¹ are used, in different proportions² according to the factories. Steamed glutinous rice is mixed with “Kōji” and “Shōchû,” in a large wooden vat. The mixtures are stirred once every two days in the beginning, and after some time once every week.

After 40–50 days the mash is pressed and the juice thus made is clarified in the settling vat. After ATKINSON³ the chemical compositions of common Nagareyama-Mirin are :—

Specific gravity	1.138
Alcohol	10.0% by weight
Dextrin	4.96%
Dextrose	30.1%
Water	54.94%

C. MARUYAMA⁴ at the Hygienic Institute of Japan analysed three samples of Nagareyama-Mirin and obtained as an average composition the following data :—

Alcohol	12.54% by weight or 15.80% by vol.
Extract	35.30%
Glucose	32.89%
Dextrin	1.84%

The same author reports as an average chemical composition of the three samples of “Mirin” from Ōsaka the following :

Alcohol	10.39 weight % or 13.09 vol. %
Extractive matters	22.69%
Glucose	20.59%
Mineral matters	0.12%

The mash of “Mirin” contains too much alcohol to cause alcoholic fermentation ; therefore the change of the chemical composition of the raw

1. “Shōchû” is a kind of spirit obtained from “Saké-kasu” or “saké.”
2. The proportion of raw materials are as follows :—

Locality.	Glutinous rice.	Kōji.	Shōchû.
Itami.	9.0 koku	3.3 koku	14.0 koku (5 shō-dori)
Ōsaka.	7.0 „	2.5 „	18.4 „ (5 shō-dori)
Nagareyama.	13.0 „	4.5 „	10.0 „ (3 shō-dori)

3. ATKINSON. Chemistry of Saké Brewing. p. 72.
4. Yakugaku-zasshi (Journal of the Pharmaceutical Society of Japan,) No. 191.

materials is due altogether to the enzyme action of Kōji-fungus, *Aspergillus Oryzae*; i.e. diastase, invertin, peptase, oxidase, or simply the enzymation. This simple process of "Mirin"-manufacture is nevertheless often a cause of great trouble to the manufacturer. In Japan, in examining the quality of "Mirin," turbidity caused by the warming (about 80° C) or by dilution with water, is objected to for the reason that such "Mirin" cannot be used for Japanese soup or in diluted and boiled "Soy." On account of this difficulty a number of years ago S. SAWAMURA made some experiments and arrived at the conclusion,¹ that the turbidity or coagulum caused by the addition of water to "Mirin" or by simply warming it, is probably on account of a compound of calcium and maltose, which is dissolved by the acid of "Mirin" and thereby appears as turbidity when the acid is diluted with water. He proposes as a prevention of such turbidity or coagulum, to prepare "Mirin" not containing lime. About twelve years have passed, but no one has made further researches and the cause of the trouble remained until now undissolved.

The results of our experiments have led us to quite different conclusions from S. SAWAMURA'S, but we do not believe that our case is the sole cause of turbidity.

**I. The nature of the substance, which causes turbidity
or coagulum when "Mirin" is diluted with a
large volume of water or by simply
warming it.**

"Mirin" was boiled in a small beaker at 80°–82° C, the coagulum formed thereby was collected on filter paper and after washing with water several times, also thoroughly washing with alcohol and ether, the residue thus obtained was examined for protein i.e. MILLON'S reaction, REICHL'S test, HOPKINS and COLE'S tryptophane-reaction, biuret-reaction. It contained 15% of nitrogen. From these reactions and the contents of nitrogen the coagulum obtained was ascertained to be a kind of protein matter. Furthermore, the

¹ Journal of the Tokyo Chemical Society, Vol. 21, p. 451,

coagulum was treated with conc. HCl and boiling a while was tested for iron by prussian-blue reaction, and the result was positive for the presence of iron. This coagulum is almost insoluble in absolute alcohol, but soluble in lactic acid or acetic acid and from such solution it is precipitated as a flocculent matter when the acid is neutralized with alkali, but re-dissolves in the excess of the reagent. Therefore, if we increase the acidity by adding lactic acid into such "Mirin," there occurs no coagulum even after warming.

The acidity, however, of "Mirin" which became turbid was stronger than normal "Mirin," indicating that the latter does not contain originally such coagulable matter. Moreover, the direct proof of the absence of such coagulable matter in normal "Mirin" was made by warming it after removal of the acidity by neutralizing with alkali.

From the above experiment, it is highly probable, that the "Mirin" which gives turbidity on warming contains a relatively larger quantity of acid at the beginning of the mash preparation. The chemical compositions of normal and abnormal kinds of "Mirin" differ as shown in the following table.

	Abnormal samples.		Normal sample.
	A	B	
Acid (as succinic acid)	0.0737%	0.0851%	0.0378%
Amino-acids (as glycocoll)	0.207%	0.224%	0.102%
Tryptophane (with Br.-water)	Plenty.	Present.	Not found.

Thus we can suppose that the abnormal "Mirin," i.e. causing coagulum on warming, is a product of advanced decomposition, more than the normal one, at least with regard to the protein matters in the raw materials.

II. First Experiment.

The object of this experiment was to determine whether the acidity of Kōji or the mash has certain influences upon the formation of the coagulable matter in "Mirin," and besides to decide, whether the temperature of the

mash at the time of the mixing of the raw materials has any influence on the formation of the same or not.

Vat.	Temp. of steamed rice at the mixing with Kōji.	Temp. of the mash after mixing the three materials.	Acidity of Kōji used (as lactic acid).
A.	55° C	21.5° C	0.525% ¹
B.	57° C	22.5° C	0.175%
C.	70° C	21.0° C	0.175%
D.	80° C	33.0° C	0.175%

PROPORTION OF RAW MATERIAL
IN A. B. C. D. VATS.

Glutinous rice.	Kōji.	Alcohol. (38 vol.%, almost equal to) "Sanshō-dori" "Shōchū."
0.085 koku	0.025 koku	0.062 koku

The treatment of the mash after the mixing was alike to that of the common factory, and after every ten days samples for analysis were taken, and after filtration the quantitative determination was made for acidity and amino-acid, coagulum on warming, and turbidity by the addition of water besides qualitatively on tryptophane. The acidity, quantity of amino-acid and the specific gravity of the samples were increased successively in every successive sampling, whereby the increase of both acids was noticeable in *A* and *C* vats, indicating that the acidity of the mash and the temperature of it at the mixing operation, have decidedly an influence upon the enzymation or ripening of the "Mirin"-mash. The presence of tryptophane was noticeable after 39 days in *A*-vat, and the coagulum on warming appeared on the same day in vats *A* and *C*.

The amino-acids amounted at the period to:—

<i>A</i>	<i>B</i>	<i>C</i>	<i>D</i>
0.269%	0.216%	0.265%	0.220%

1. The acidity of this kōji was artificially increased by the addition of lactic acid.

After 49 days the samples from all vats exhibited an evident reaction for tryptophane, but only traces in the *B* and *D* vats. The coagulum on warming was observable in all cases, but turbidity caused by the addition of water was noticeable with the samples *A* and *C*, while *B* and *D* gave no such change. The acidity and amino-acids by this time were as follows:—

	<i>A.</i>	<i>B.</i>	<i>C.</i>	<i>D.</i>
Amino-acids (as glycocoll)	0.307%	0.215%	0.286%	0.227%
Total acid (as lactic acid)	0.135%	0.109%	0.115%	0.106%
Coagulum	0.900%	—	0.06%	—
Spec. grav.	1.133%	1.134	1.136	1.133

The analyses were made twice after this period and the quantity of acids, amino-acids, tryptophane, turbidity and coagulum increased by degree.

From the above mentioned facts the coagulum on warming begins to appear when the quantity of amino-acids reaches above 0.227% as glycocoll, or the quantity of the acid above 0.115% as lactic acid; and in every case when coagulum appears in “Mirin” on warming, there occurs surely tryptophane. After 110 days the mash was pressed and after filtration the analysis was made with the following results:—

Sample from vat.	Specific gravity.	In 100 c.c. of “Mirin.”				
		Coagulum on warming.	Total acid as lactic acid.	Amino-acid as glycocoll.	Glucose.	Dextrin.
<i>A.</i>	1.142	0.7851 gr.	0.5330 gr.	0.6727 gr.	40.00 gr.	1.929 gr.
<i>B.</i>	1.143	0.2678 „	0.4311 „	0.5320 „	39.33 „	5.780 „
<i>C.</i>	1.1415	0.6144 „	0.4430 „	0.6152 „	37.46 „	2.586 gr.
<i>D.</i>	1.41	0.7554 „	0.3672 „	0.5242 „	38.68 „	4.514 „

III. Second Experiment.

In the above experiment, we have confirmed that the acidity of Kōji and Shōchû i.e. the acidity of the mash, has a decided influence on the formation

of the turbidity or coagulum, in this experiment the acidity was reduced by neutralizing with carbonate or by decreasing the amount of Kōji. Also, a comparative study was made about "Waka-Kōji," young, and "Ci-Kōji," old Kōji.

Vat.	Koji.	Acidity of Koji.	Remarks.
I.	young	0.227%	The acidity mentioned was obtained by the addition of lactic acid to young Kōji:—The acidity is equal to old Kōji. This acidity is equal to five times that of young Kōji, made by the addition of lactic acid. <i>NaCl</i> is added to make 0.01% against "Shōchū."
II.	"	0.915%	
III.	"	0.181%	
IV.	"	0.181%	Control.
V.	old	Neutralized with Na_2CO_3 .	The acidity of "Kōji" is equal to the control, but the amount of Kōji used was reduced to half of the control. Control.
VI.	"	Neutralized with $CaCO_3$.	
VII.	"	0.227%	
VIII.	"	0.227%	Control.

The temperature of the room was warmer¹ than in the first experiment, so that after 26 days the coagulum and tryptophane appeared in the filtrate of the mash, except in vats V and VII. The turbidity by the dilution with water was observable even in the case of vats V and VII. The results of the analyses are shown in the following table:—

Samples from vat.	Sp. grav.	Tryptophane	Coagulum on warming.	Turbidity by dilution.	Free acid as lactic acid.	Amino-acid as glycocoll.
I.	1.144	Strong	Appear.	Evident.	0.198%	0.286%
II.	1.153	Evident, but less than I.	Appear.	Evident.	0.327%	0.440%
III.	1.143	Evident almost same as II.	Appear.	Evident.	0.181%	0.286%
IV.	1.145	?	Trace.	Evident.	0.164%	0.263%
V.	1.132	No reaction.	None.	Trace.	0.150%	0.192%
VI.	1.143	Present.	Appear.	Evident.	0.249%	0.295%
VII.	1.133	No reaction.	None.	Trace.	0.126%	0.132%
VIII.	1.135	Present.	Appear.	Evident.	0.212%	0.277%

1. It was 20–23° C during this experiment.

From the above table it will be seen that the *coagulum* formed on warming goes parallel with the decomposition of protein, i.e. the quantity of amino-acids; but the *turbidity* occurring by the dilution with water must be a different matter, inasmuch as this difficulty appears relatively quicker¹ than the former in the stage of less amino-acid, namely even in absence of tryptophane.

After 51 days the mash was pressed and clarified, giving the following analytical data:—

Samples from vat.	Specific gravity.	Coagulum. in 100 c.c.	Free acid as lactic acid. in 100 c.c.	Amino-acid as glycocoll. in 100 c.c.	Glucose. in 100 c.c.	Dextrine. in 100 c.c.
I.	1.155	0.307 gr.	0.350 gr.	0.648 gr.	38.13 gr.	0.991 gr.
II.	1.1695	0.544 „	0.535 „	0.909 „	40.10 „	1.469 „
III.	1.1610	0.177 „	0.331 „	0.581 „	38.13 „	0.323 „
IV.	1.1625	0.175 „	0.265 „	0.472 „	39.23 „	0.693 „
V.	1.1495	0.192 „	0.203 „	0.381 „	38.49 „	1.715 „
VI.	1.1595	0.444 „	0.365 „	0.612 „	37.95 „	1.496 „
VII.	1.1605	0.130 „	0.208 „	0.345 „	37.77 „	0.312? „
VIII.	1.1605	0.481 „	0.317 „	0.593 „	39.23 „	0.346 „

The third experiment was carried on with regards to the comparison of K_2CO_3 , Na_2CO_3 and $CaCO_3$ in the application of neutralization of the acid in the mash. The fourth experiment was on the influence of the temperature of the steamed rice to be cooled, or the temperature of the mash when the mixing operation is finished. The result was that the temperature at the mixing operation has a great influence in inhibiting the peptonifying action or more strictly the amidisation process of the protein:—the higher the better, and 40–45° C as a highest limit. Among the carbonate used for the neutralization, sodium-salt was the best. The varieties of *Aspergillus Oryzae* behave differently in this process, some decomposing protein rather quickly, but the others slowly and in less degree.

1. This phenomenon is contradictory to the first experiment, therefore we must proceed still further on this point.

Conclusion.

The ripening process of "Mirin" mash is due to a mere enzymation: the saccharification and amidisation or protein decomposing process. Some inferior kind of "Mirin" causes turbidity when it is diluted with a large quantity of water, or forms coagulum when warmed to about 80° C. The coagulum is a kind of protein containing 15% of nitrogen, soluble in organic acids, especially in lactic or acetic acid but is precipitated by dilute mineral acids:— HCl , HNO_3 , H_2SO_4 . The cause of the occurrence of such partly decomposed protein matter in "Mirin" is too much acidity of the mash, derived from "Shōchū" and "Kōji":—*the acid accelerates the enzymation and decomposes protein beyond limit.* The reduction of the acidity by the use of Na_2CO_3 gave a favorable result, but the temperature of the mash in its mixing operation should be carefully watched and the varieties of *Aspergillus Oryzae* must be well selected to prepare constantly good and normal "Mirin."

On Bacillus Natto.

BY

Shin Sawamura.

Natto is an article of food prepared by leaving boiled soy-beans wrapped in rice straw in a warm place for a night, and thus making them ferment. Soy-beans of natto are coated with a characteristic slimy substance. The author¹ separated formerly two species of bacilli from natto obtained in Tokyo, No. I of which produced good flavored natto when inoculated to boiled soy-beans, and No. II strongly slimy one. The former bacillus was considered to be the chief actor in natto fermentation and received the name of "*Bacillus natto*." In later years the author examined bacteriologically many samples of natto obtained at various localities, and found that the producer of natto is the same in all cases, viz. "*Bacillus natto*." This bacillus can produce natto of good flavor and strong viscosity, and the presence of other microbes is not necessary in the fermentation of natto. The bacteriological description of *Bacillus natto* is as follows.

Form: The bacillus measures 1 μ in width and 2-3 μ in length. The ends of the rod are round and the bacilli unite in two or more.

Mobility: Motile.

Spore-formation: The bacillus forms a spore mostly in the middle of the cell.

Gram's method: It is not decolorized by GRAM's method.

Oxygen: Aerobic.

Bouillon: It produces a light brown, thin, characteristic dry mealy scum which is broken into pieces by shaking. Bouillon does not become turbid.

1. Bulletin of Agricultural College, Tokyo. Vol. VII. p. 107.

[Jour. Coll. Agric., Vol. V, No. 2, 1913.]

Pepton water: The scum formation is the same as in bouillon, but its color is lighter.

Agar plate culture: The colony is light brown and flat, and has a characteristic dry mealy appearance, with a small point in the centre of the colony, the periphery of which is irregular and divided feather-like. It produces a smelling of natto.

Gelatine plate culture: Small colonies are formed which liquefy gelatine quickly.

Agar streak culture: It produces a light brown flat and characteristic dry mealy colony.

Gelatine streak culture: It is quickly liquefied along the needle track.

Agar stab culture: It grows only along the needle track and forms no branch.

Gelatine stab culture: It is liquefied along the needle track.

Soy-bean agar: The colony has folds and is rougher than on bouillon agar.

Potato: Gray slimy colony with many folds, resembling that of potato-bacillus.

Gas: It is not evolved in glucose bouillon.

Azolithmin-milk: It is reddened at first, and then decolorized, and the milk becomes clear. After many days azolithmin turns somewhat blue when it is newly added.

Indol: Pepton water culture kept at 32° C for 7 days gives the characteristic reaction, neither with NaNO_2 and H_2SO_4 , nor with nitroprussic acid and NaOH .

H_2S : It is not formed.

It was confirmed by the previous investigation that *Bacillus natto* produces a trypsin-like enzyme, and decomposes protein of soy-beans. In order to know how protein is decomposed by this bacillus, boiled soy-beans were inoculated with this bacillus and kept at 35° C, one sample for 14 hours and the other for 7 days. The viscosity and flavor of natto thus prepared are stronger in the younger than in the elder.

The nitrogen in various forms and soluble organic matter in the percentage of dry matter were found to be as follows:—

	14 hours	7 days
Total nitrogen	7.363	7.421
Insoluble albuminous nitrogen	5.881	2.104
Soluble „ „ 	1.482	5.317
„ coagulable „ 	0.307	0.182
„ uncoagulable „ 	0.321	0.477
Nitrogen of pepton and polypeptides	0.208	0.408
„ „ arginin, histidin and lysin	0.069	0.085
„ „ purin bases	0.086	0.140
„ precipitated by phosphotungstic method ..	0.109	2.111
Soluble organic matter	21.947	41.546

Soy-bean contains nitrogen chiefly in the form of protein (85-90%), non-albuminous nitrogen being very little (10-15%).

Bacillus natto produces diastase, but reducing sugar was not found in natto thus prepared. This is probably due to the fact that soy-beans do not contain much starch, the main part of carbohydrate being in the form of galactan, &c, wherefore the little glucose formed was decomposed again by the bacillus.

The author expresses hereby sincere thanks to Mr. OSHIMA, assistant of the College, who analysed natto as above mentioned.

Preliminary Notes on the Chemical Composition of "Miso."¹

BY

Teizō Takahashi and Gorō Abé.

"Miso" is a food very common in Japan. The raw materials used in making it are soy beans, rice or barley, common salt, and water. In preparing "Miso," rice or barley is changed at first into "Koji," and the boiled soy-beans, common salt and water are mixed and put in a cool place to ferment slowly. The duration of ripening differs according to the varieties of "Miso."

The Japanese consume it daily as a sort of food adjunct to vegetables. Statistics on the consumption or the output do not exist at present, as it is made by the families themselves and only in large cities or in certain localities special "Miso" factories are established. Assuming, however, 10 momme (37.5 grams) to be the least quantity daily consumed per head and that 30 millions out of the 50 millions of the whole population eat "Miso" every day, the annual consumption of "Miso" would amount to nearly 45 million kilograms.

On the chemical composition of "Miso," O. KELLNER, M. NAGAOKA,³ and S. KURASHIMA (2) made elaborate researches, but they overlooked amino-acids.

The authors have made preliminary researches with three varieties of "Miso," in order to decide which variety contains the largest amount of amino-acids and would, therefore, be the most suitable sample for the isolation of the acids.

1. This report is already published in the Journal of the Tokyo Chemical Society, Japan, Vol. 29, No. 2, Feb. 1908.

2. Researches on the manufacture and composition of "Miso." Bulletin of the Agric. College, Tokyo. Vol. I, No. 6, p. 2—24.

3. Bulletin of the Agric. College Tokyo, Vol. I, No. 5, 1895.

[Jour. Coll. Agric., Vol. V, No. 2, 1913.]

The results are tabulated below :—

(grams in 100 parts of fresh substance.)

	Hatsuchō-miso.	Sendai-miso.	Inaka-miso.
Water	46.873	50.933	51.410
Ash	13.794	12.816	12.213
Total— <i>N</i>	1.932	1.835	1.645
soluble in water, therein	1.707	—	1.238
Protein— <i>N</i>	1.070	0.945	0.9115
soluble in water, therein	0.9157	—	0.8923
Organic bases	0.627	0.513	0.5228
Ammonia— <i>N</i> . (WURSTER'S method) ..	0.0237	—	0.0216
Other— <i>N</i>			
chiefly mono-amino-acids	0.297	—	0.1891
Fat	6.208	—	5.892
Chlorine	6.105	—	—
(As <i>NaCl</i> .)	11.544	—	—
Alcohol	Trace.	—	—

From the above table we can easily see that “Hatsuchō-miso” was most suitable for our purpose and therefore further researches were made with this variety. The component of “Hatsuchō-miso” was recalculated to the dry matter of the sample:—

Ash	26.77 %
Organic matter	73.33 „
Total— <i>N</i>	3.77 „
Protein— <i>N</i>	2.089 „
Soluble in water, therein	1.788 „
Non-albuminoid— <i>N</i>	1.683 „
Organic base— <i>N</i> + peptide— <i>N</i>	1.224 ¹ „
Ammonia— <i>N</i> . (WURSTER'S method)	0.046 „
Other— <i>N</i> . (chiefly mono-amino-acids)	0.410 „

1. This nitrogen is determined in the precipitate formed by phosphotungstic acid after the removal of protein. This precipitate contained *peptide* beside organic bases. The evidence of the fact becomes very clear, when we examine the following two cases

	Nitrogen per 100 parts.
P.pt. by phos.-tung acid after removal of protein	0.5673
P.pt. by phos.-tung. acid after removal of protein and after digestion for eight hours with 20% <i>HCl</i>	0.3268

I. Organic Bases.

One kilogram of the sample was macerated in a mortar with the addition of water, and warming gently was filtered. The operation was repeated and after the removal of protein matter by basic lead acetate, di-amino-acids or bases were isolated according to the method of KOSSEL and KUTSCHER.

a) PRECIPITATE FORMED BY MERCURIC CHLORIDE.

After the removal of mercury by H_2S -gas, the fluid was evaporated under reduced pressure, but we could not find the characteristic crystals of chloride of histidine. The crystals consisted chiefly of quadratic plates and gave PAULY'S diazo-reaction, indicating the existence of a trace of histidine. In recrystallizing the crystals, we obtained fine needles, fine plates, and octahedrons, the latter gave as melting point $190-195^\circ C$ (uncorr.). From this chloride picrate was prepared, which was a mixture of crystals in the shape of needles, plates, and prisms, and the former crystals gave their melting points as follows:—

- 1) Contraction occurred at $245^\circ C$ and burst at $256^\circ C$ (uncorr.).
- 2) Contraction at $240^\circ C$ and decomposed at $242-243^\circ C$ (uncorr.).

The methyl-ester prepared from the chloride recrystallized from ether, melted at $212^\circ C$ (uncorr.).

b) PRECIPITATE FORMED BY SILVER-NITRATE AND BARYT WATER.

From this precipitate a base was prepared and the picrate therefrom gave a melting point of $193^\circ C$ (uncorr.), therefore there exists too wide a difference of degree in comparison to that of true arginin picrate ($212^\circ C$). The substance was too small to determine accurately, whether this base is an isomer of arginine or other base.

c) PRECIPITATE MADE BY PHOSPHO-TUNGUSTIC ACID.

From this precipitate a base was found which gave a needle shaped picrate, without melting even at $360^\circ C$. It may be picrate of ammonia, but NESSLER'S test failed to prove it, moreover, the material was too small to identify the base.

II. Mono-amino-acids.

Mono-amino-acids were isolated by the well known ester method of E. FISCHER'S; the quantity of "Miso" used for this purpose was one kilogram. The fractional distillation was carried out under the reduced pressure of 15—18 m.m. and the fractions obtained were:—

1) Below 40° C	5.5 g.
2) 40°— 60° C	1.2 „
3) 60°—100° C	4.0 „
4) 100°—130° C	6.2 „
5) 130°—200° C	2.0 „

In the second case the substance taken was 1.75 kilograms and the fractions of the ester obtained were:—

1) 40°— 60° C	4.2 g.
2) 60°—100° C	5.3 „
3) 100°—152° C	3.5 „
5) 152°—200° C	1.3 „

Glycocoll was not found, but alanine was identified in the first fraction (1.5 g.) of the first series. It tasted very sweet and melted at 260° C (uncorr.). The same compound was found in the second fraction of the first series (0.1 g.), which melting at 260° C gave the following nitrogen contents:—

Substance taken: 0.1240 g.

Nitrogen: 15.5 c.c. (at 12° C, and 760 m.m.)

Calculated for $C_3H_7ON_2=N$. 15.67%

Found. =N. 14.87%

Proline was obtained in the fraction of 40°—60° C in both cases; but only a trace in the first series, 0.1 g. in the second series. It tasted bitter. Moreover, the third fractions, too, contained (0.3 g) proline. The quantity was too small to allow working on elementary analysis, but the characteristic property that this substance dissolves in absolute alcohol is sufficient to identify the compound. The copper salt of this acid prepared by copper hydrate contained active and rasemic components, one of which dissolving in absolute alcohol, others not.

Leucine was found in the fraction of 60°—100° C in both series:—1.04 g. in the first series and 3.5 g. in the second series. It was snow-white and tasteless, melted at 293°—295° C (uncorr.), and in *HCl*-solution (20% *HCl*) gave leavo-rotation.

Substance taken 0.079 g. in *HCl* (20%) solution.

$$[\alpha]_D^{20} = -18.72^\circ.$$

The dried substance yielded:—

Substance taken. 0.1016 g.

*Co*₂, 0.1975 g. Water 0.0859 g.

Substance taken. 0.0928 g.

N. 9.5 c.c. (at. 13° C, 752 m.m.).

Calculated as *C*₆*H*₁₃*N**O*₂ Found.

C. 54.96% 53.26% *H*. 9.93% 9.425% *N*. 10.68% 10.83%

Leucine was also obtained from 100°—130° C or 130°—200° C fraction. It tasted somewhat bitter and sweet and amounted to 1.5 g. melting at 265°—270° C (uncorr.). On analysing it yielded 10.42% of nitrogen.

Calculated for *C*₆*H*₁₃*N**O*₂ *N*=10.68% Found. *N*=10.42%

From the same fraction which gave leucine was obtained aspartate of barium, but the amount was too small to identify it.

From the filtrate of aspartate, sodium salt of an amino acid was prepared, which crystallised prismatically and melted at 210° C (uncorr.). It is highly probable that this salt is glutamate, but the quantity was insufficient for identification.

Tyrosine was prepared from 500 g. of "Miso" from the filtrate of protein precipitation. It gave an intense red color by MILLON's reagent, and PAULY's diazo-reaction.

Cystine. It was proved too in the filtrate in which tyrosine was tested.

Above all, we have observed that the taste of "Miso" is quite independent from the protein substance in it, namely all tastes of "Miso" are contained in the filtrate of the precipitate of the protein.

5. In August 1903, KIKUNAE IKEDA found that the salt of glutamic acid tastes very similar to bonito, so we have reason to believe that a part of the taste of "Miso" must be ascribed to glutamate contained in it.

Summary.

From the above analysis, it may be seen that one kilogram of "Miso" at least contains ;

Alanine	Ca. 2. g.
Leucine	„ 7. g.
Proline (active and racemic)	0.3g.
Phenylalanine	Not found
Aspartic acid	Trace
Glutamic acid	Ca. 0.5 g.
Histidine	Trace
Arginine	?
Lysine	0.31 g.
Tyrotine	Trace
Cystine	Trace
Albumoses	Present
Peptones	Present

Observations on the Microorganisms of the Mash of "Shaoshing-chu" and "Chu-ya."

BY

Teizo Takahashi.

With Plates IX—XIV.

Introduction.

"Shaoshing-chū"¹ or "Lau-chū"² is one of the most liked alcoholic beverages in China, chiefly manufactured at Shaoshing and Kang-chou in the province of Chū-chiang. The annual output is not accurately known but the amount manufactured in the two above mentioned towns is estimated roughly at 40,000 hectolitres.

The raw materials used for the manufacture of Shaoshing-chū are boiled glutinous rice, "Chū-ya",³ "Chiu-tzó"⁴ and water.

These materials are mixed in large china vessels, in which they are fermented for one month or longer, after which time the mash is filled into linen bags and pressed. The clarified fluid is pasteurized by means of a water bath in a special apparatus made of tin, and poured into small china vessels with a small opening, which is covered by a dish-shaped earthen ware cover, over which clay is heaped up to 5-7 inches in height to seal and make the interior of the vessel air-tight. The common "Shaoshing-chū" in the market is that which has been aged three years, only

1. "Shaoshing-chū" is derived from the name of the town, where the beverage is chiefly manufactured, and "chū" means alcoholic drink.

2. In "Lau-chū", "Lau" means "old", because this beverage is stored very long, at least 3 years, for the purpose of ageing.

3. "ya" means "medicine". A similar substance to "Chū-ya" is described as "*Chinese yeast*" by CALMETTE, VODERMANN, WEHMER and others.

4. "Chiu-tzó" is a fungus mass prepared from barley.

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a few people in China, rich or of high rank, are accustomed to drink the special article which has been kept in stock for some ten or twenty years.

The mash and "Chū-ya"⁵ used for the investigations were brought from China by the writer, and the former was of special importance for the purpose; for it was taken from a factory at a village named Tong-pow near Shaoshing, in a flask *previously sterilized*.

On the "Chū-ya" of "Shao-hing-chū" no mention has been made as yet in literature, but a similar substance, or rather the same material, is described as "Chinese yeast" by many observers. The first was CALMETTE⁶ and his material was brought from Saigon. On the "Ragi," from Java, VODERMANN⁷ writes already in 1893 and in the next year this was followed by ELJKMANN'S⁸ report on the Chinese yeast of the arak manufacture in Batavia. In 1895 WENT and PRINSEN GEERLIGS⁹ widened the field of investigation and studied the yeasts as well as the sugar forming moulds, and found a new species of *Mucor*. DELBRÜCK'S¹⁰ observation on the physiology of fermentation with *Amylomyces Rouxii* or *Mucor Rouxii* of CALMETTE followed subsequently. On Chinese yeast of Singapore¹¹ and Javanese Ragi,¹² WEHMER'S report must not be neglected as an allied and detailed description.

Mucor Cambodja, *M. Rouxii* were found in the Chinese yeast of Saigon and Cambodja by T. CHRZASZEZ,¹³ while *Rhizopus Chinensis* and *Rhiz. Tritici* were found as new species beside *Aspergillus glaucus*, *Asp. flavus*, *Mucor racemosus*

5. The best kind of "Chū-ya" is said to be prepared in Ning-pow. The writer's sample was purchased in Shaoshing and Kang-chou.

6. Ann. de l'Institut. Pasteur. 1892. T. VI. La levure Chinoise.

7. VODERMANN. (Analecto op bromatologisch gebied. I. Geeneskundig Tijdschrift voor Nederl. Indië. D. XXXIII. (1893. p. 359. ff.)

8. ELJKMANN: Mikrobiologie über die Arakfabrikation. Batavias, Centb. f. Bakt. Bd. XVI. 1894. p. 99.

9. WENT u. PRINSEN GEERLIGS: Beobachtungen über Hefearten u. Zuckerbildende Pilze der Arakfabrikation. (Verhandlungen d. Konigl. Akad. van We eus. te Amsterdam. 2. Ser T. IV. No. 2. 1895).

10. DELBRÜCK: Zeit. f. Spiritus Indust. 1898 u. 1899.

11. WEHMER: Die Chinesische Hefe u. der sogenannte *Amylomyces*. Centb. f. Bakt. 1900. VI. 11. S. 353-365.

12. WEHMER: Der javanische Ragi u. seine Pilze. Centb. f. Bak. 1900. Bd. VI. S. 610-619.

13. T. CHRZASZEZ. Centb. f. Bakt. Bd. VII. S. 326-338. 1901.

and *Penicillium glaucum* in "Chiu-tzó"¹⁴ of Shao-shing by K. SAITO¹⁵ (1904). In the next year, he studied the "Tzó-zu"¹⁶ from Shan-tung (China) and a new species of *Rhizopus*:—*Rhiz. oligosporus* was isolated beside *Rhiz. chinensis* and a *Sachsia* sp.

So far, the observation of the microorganisms of Chinese yeast was chiefly limited to the mould fungus; the writer proceeded to isolate *Saccharomyces* from "Chū-ya" (Chinese yeast, Shaoshing). The form of "Chū-ya" is oval and greyish colored, so that it resembled rather Javanese Ragi after WEHMER. Its main part consists of glutinous rice, but the microbes in it seem to differ according to the place, where it is made. From the sample from Shaoshing, the microbes isolated are *Mucor*, *Penicillium glaucum*, *Chalara*, *Willia anomala* and a *Tetradé*, while from the sample from Kang-chow *Mucor*, *Willia anomala* and sometimes *Saccharomyces* were isolated by plate culture.

On the microbes of the "Shaoshing-chū"-mash, no mention has hitherto been made in the literature. The yeast varieties isolated are described below, and the special observation on the spore culture and its germination made by Mr. K. MASUNAGA is also quoted therein.

Part I. *Saccharomyces* Group.

I. *SACCHAROMYCES* SHAOSHING VAR. I.

1. Cell and colony: The forms of the colonies in "koji"-extract gelatine (42 days at 15–16.5°C.) are round with a superficial elevation at the central part and around which many concentric ring markings are visible. The margin of these colonies terminates with fine radiated streams. These characters remind us of the natural gigantic colonies of A No. 6 and B No. 15 of saké yeast.

14. "Chiu-tzó", as mentioned before, is prepared from barley.

15. K. SAITO: Centb. f. Bakt. II Abt. Bd. VIII. 1904. No. 517. S. 153–161.

16. "Tzó-zu" is made from rice and is used as a raw material in the manufacture of an alcoholic beverage at Shang-tung (China). It plays a like rôle as "Chiu-tzó" and is a substance quite different from so-called "Chinese yeast."

The forms of the cell are round, short elliptic, or oval. The elliptic spores (2.5μ) are found in old cells (50 days culture in "koji"-extract gelatine) 2 or 3, sometimes 4 in number, and the size of the cells at the time measures $7.5 \times 7.5\mu$, $7.5 \times 5\mu$, $7.5 \times 6.8\mu$, $6.8 \times 5.8\mu$; or $7.5 \times 6.3\mu$, $6.8 \times 5\mu$, $6.8 \times 6.8\mu$, $6.2 \times 4.2\mu$. In very old culture (6 months surface culture in "koji"-extract agar) there are found cells with 1-4 spores which are chiefly elliptic in shape but sometimes they are round when the ascus is composed from four spores. The spore formation at 25°C was perceived after 19 hours on the gypsum block, after 21 hours in pure agar, 24-40 hours in Gorodokowa's agar, 4-5 days in diluted "shōyu," 14 days in "koji"-extract-agar.

2. Growth: *Surface culture*. In "koji"-extract gelatine (22 days at $15-16^\circ\text{C}$. cf. the plate) the growth appears as a white, almost lustreless waxy covering with some elevation, and the margin ends in streams. The surface growth in glucose-saké-agar (14 days at $20-21^\circ\text{C}$.) is rather smooth, elevated at the central part, with somewhat wide streams at the margin. (cf. the plate.) The growth in "koji"-extract-agar (25 days at $22-28^\circ\text{C}$. in the test tube) is colored light yellow, while the sediments in the condensed water has a slightly *rosy tint*. The denser yellow coloration is perceivable in the covering with the same medium after 16 days culture at $21-23^\circ\text{C}$, after two months it changes into a brown color. The growth in saké-agar (18 days at $20-21^\circ\text{C}$.) has a white and smooth surface, but a rosy red coloration becomes evident at the thick part. In the glucose (2%)—saké-agar (11 days at $21-22^\circ\text{C}$.) the covering appears as a more or less rosy colored pasty mass, while the coloration retarded after further 3 days culture at 19.2°C . and recovered after four days at 20.5°C . In the same medium, the growth at $33-34^\circ\text{C}$ after 16 days' culture still kept its rosy coloration. *Fluid culture*: In "koji"-extract (2 weeks at $18.2-19^\circ\text{C}$.) there settles a greyish sediment in a clear fluid and the yeast ring hangs at the wall of the test tube as a semitransparent mass.

3. Behavior toward sugars: By LINDNER's small fermenting method¹⁷ the behavior toward the sugars was found:—

Glucose.	Galactose.	Maltose.	Saccharose.	Raffinose.	Lactose.	Dextrin.
+++	+	+++	+++	+++	—	—

17. Wechensch. f Brauerei. 1911. No. 6. "Kleingährmethode".

4. Fermentation products: The culture in “koji”-extract (12°B) in ERLÉNMEYER'S flask gave the products below:—

	Alcohol.	Ester.	Fusel oil.
A. 7 days at 21–22°C.....	5·0 vol%	————	present
B. 10 „ „ „	4·93 vol%	————	„
C. 20 „ „ 28°C.....	3·42 vol%	0·264% ¹⁸	„

Among the fine aromas of the esters formed, the flavour of acetacetic ester predominated. In the fusel oil, beside amylalcohol the presence of isopropylalcohol was highly probable. The formation of organic acids and the assimilation of the amino-acids were examined with the same medium giving the data below:—

		Amino-acids, as glycocoll.				Organic acids, as succinic acid.		
		In	After	Assimilat	Coeff. of	In	After	
		origin. sol.	growth.	-ed.	assimilation.	origin. sol.	growth.	Formed.
		%	%	%		%	%	%
I.	7 days at 22–25°C.....	0.1391	0.0744	0.0647	46.5	0.0344	0.170	0.136
II.	7 days at 29–30°C.....	0.1270	0.0839	0.0431	33.0	0.0328	0.116	0.0840
IV.	29 days at 16–19.5°C.....	0.2329	0.1650	0.0679	29.4	0.0708	0.144	0.0740
V.	10 days at 24.5–25.5°C....	0.2560	0.0593	0.1967	76.0	—	—	—
VI.	10 days at 28°(33°)C. ¹⁹	0.1590	0.0760	0.083	53.0	0.0611	0.115	0.0540
VII.	20 days at 28°C.....	0.2560	0.1460	0.110	43.0	0.0620	0.109	0.0740

The culture was shaken regularly twice every day during fermentation in the experiments V to VII, while in other cases it was done irregularly. From these results, it is evident that the assimilability of the amino-acids varies according to the mode of cultivation, and the temperature of the medium. The coefficient of the assimilation of amino-acids varies from 29·4 to 76, but we have no means of reaching any conclusion whatever except in V and VII; for amino-acids in the original solution vary almost in every case. In comparing the cases V and VII, this yeast seems to have the optimum assimilation for the amino-acids near 24–25C.

II. SACCHAROMYCES SHAOSHING VAR. II.

1. Cell and colony: The forms of the colonies in “koji”-extract-gelatine (42 days at 15–16.5°C.) are round with the elevation granulated at the central

18. In this case the density of “koji”-extract used was 13°B.

19. At the end of the culture the temperature changed to 33°C.

part, while the margin part shows light but wide streams. These characters are very similar to B.7 and B.11 of saké yeast.

The cell forms are chiefly short or long ellipses, round cells are seldom found. The elliptic spores are found in the old culture (50 days in "koji"-extract-gelatine), chiefly in the elliptic cells. The size of the cells in this culture measures $6.7 \times 6.2\mu$ (long ellipse), $6.2 \times 7.5\mu$ (elliptic cell with spores,) $4.2 \times 2.5\mu$ (short ellipse), $7.5 \times 5\mu$ (deformed ellipse with spores), $5 \times 5\mu$, $2.5 \times 5\mu$ (ellipse). From two to four round or elliptic spores are found in the majority of the ascus in the old culture of "koji"-extract-agar (6 months culture), and when two spores are present in an ascus they are more or less longer than those where three occur in number. On the gypsum block the spores are found after 19 hours at 25°C . and in the other media the duration of the spore formation is quite the same as in var.I, except in "koji"-extract-agar (6-7 days) and diluted "koji"-extract (1-6 days).

2. Growth: The *surface culture* in "koji"-extract-gelatine (22 days at $15-16^{\circ}\text{C}$.) has a similar appearance with the former species but differs in having more lustre than the former and wider streams on the margin. (cf. the plate). The same culture in "koji"-extract-agar (14 days at $20-21^{\circ}\text{C}$.) has a growth similar to the former one, but the streams on the margin are lighter (cf. the plate). The surface culture in "koji"-extract-agar in the *test tube* (25 days at $22-28^{\circ}\text{C}$.) is white with a very slight yellow tint, but the surface is quite smooth and by this is distinguished from the former. The similar culture at $21-23^{\circ}\text{C}$. (16 days) begins to color light yellow after 8 days and changes to brown like the former species. In saké-agar (18 days at $20-21^{\circ}\text{C}$.) the surface growth is generally white, so the rose coloration is hardly perceivable even at the thick part of the growth and by this character is distinguished from the former. Another character differing from the former one is the *white* waxy covering formed in glucose-saké-agar (11 days at $21-22^{\circ}\text{C}$.) and a *slight dirty brown* coloration of the surface growth in the same medium after 16 days at $33-34^{\circ}\text{C}$.

Fluid culture: In "koji"-extract (12°B .) in the test tube (two weeks at $18.5-19^{\circ}\text{C}$.) a greyish sediment was formed under the clear fluid, adding a smooth yeast ring along the wall of the tube. The yeast ring quickly formed at $25-26^{\circ}\text{C}$. in the same medium;—after 6 days.

3. Behavior to sugars: The fermenting character is shown below.

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	++	++++	+++	—	+++	—

4. Fermentation products: The mode of cultivation and the conditions necessary are equal with those in the former case.

	Alcohol.	Esters.	Fuseloil.
A. 7 days at 21–22°C.....	3.69 vol %	————	present.
B. 10 „ „ „.....	5.86 vol %	————	„
C. 20 „ 28°C.....	3.42 vol %	0.2702%	„
(as etheylacetate.)			

The flavour of the acetacetic ester was also perceivable in this culture. The formation of alcohol of this species is almost equal to the former species, but the period of the fermentation is longer in this species than in the former. The formation of organic acids and the assimilation of the amino-acids in “koji”-extract were found:—

	Amino-acids as glycocoll.				Organic acids as succinic acid.		
	In orig. sol.	After growth.	Assimi- lated.	Coeff. of assimi- lation.	In orig. sol.	After growth.	Formed.
	%	%	%		%	%	%
I. 7 days at 22–25°C.....	0.1391	0.0863	0.0428	30.8	0.0344	0.236	0.202
II. 7 days at 29–30°C.....	0.1270	0.0990	0.0280	22.1	0.0328	0.111	0.0792
IV. 29 days at 16–19.5°C.....	0.2329	0.1280	0.1049	44.5	0.0708	0.0824	0.0116
V. 10 days at 24.5–25.5°C.....	0.2560	0.0610	0.1150	45.0	—	—	—
VI. 10 days at 28°(33°)C.....	0.1590	0.0530	0.1060	66.6	0.0611	0.0324	0.032
VII. 20 days at 28°C.....	0.2560	0.1640	0.092	36.0	0.0620	0.0960	0.0340

The absolute quantities of the amino-acids assimilated are largest in the experiment V, where the temperature of the cultivation was 24.5–25.5°C as we have seen in the former species. The coefficient of the assimliation of amino-acid is generally small in this species:—from 22.1—66.6.

III. SACCHAROMYCES SHAOSHING. VAR III.

1. Cells and colonies. The form of the colonies in “koji”-extract-gelatine (42 days at 15–16.5°C.) is round with a small concave at the central

part and marked streams on the margin. The form thus described reminds us of the natural gigantic colony of saké yeast A.30. The majority of the cells are elliptic in form, but long ellipses, oval, and club shapes occur frequently. The spores are found, in the "koji"-extract-agar culture, already after 60 days and the cells holding them increase in a further longer culture:—6 months. From two to four spores are found in a cell. Their forms are round or elliptic and these two forms exist in one cell. When four spores are found in the same cell, they are generally elliptic but their sizes are unequal. They were not found in the culture of the "koji"-extract-gelatine after 50 days and are thereby distinguished from the two above mentioned species, where the size of the cells measures $4.2 \times 3.7\mu$ (ellipse), $6.2 \times 42\mu$ (ellipse), $5 \times 5\mu$, $2.5 \times 3.7\mu$ (small ellipse).

On the gypsum block the spores¹⁰ are found after 19-20 hours at 25°C. They stain well by ZIEHL's carbolfuchsin solution. The germination of the spores is of the most common type.(cf. the plate.)

2. Growth: The *surface culture* in "koji"-extract-gelatine (42 days at 15-16.5°C.) has almost the same appearance as the first (cf. plate) species. On the other hand, the culture in glucose (2%)—saké-agar shows basket-like folds on the surface with beautiful streams on the margin, (14 days at 20-21°C.), and is thereby distinguished from the two above described species. (cf. the plate). The coverings of the culture in "koji"-extract-agar (*test tube*, 25 days at 22-28°C.) have a smooth surface of yellowish-white tint with fine streams on the margin and in condensed water a dirty greyish rose colored deposit settles. The culture in the same medium after 60 days at room temperature remains waxy white against the brown colored coverings of the varieties I and II. In saké-agar (18 days at 20-21°C.) light yellow folded coverings begin to appear in contrast to the varieties I and II. The further difference from the species described consists in giving a dense rosy greyish growth with basket-like folds in glucose-saké-agar (11 days at 21-22°C.), and a dense rosy brown covering in the same medium. (16 days at 33-34°C.).

20. Spore culture at 25°C. in diluted "shōyu" succeeded after 9-12 days, in "koji"-extract-agar after 14 days, but failed in pure agar after 70 hours, in GORODOKOWA's agar after 70 hours.

Fluid culture: In "koji"-extract in the test tube a greyish sediment settles under the clear fluid after two weeks at 18.5–19°C, and a thin yeast ring and island over the surface of the fluid begins to form. The yeast ring formation ensues after 6 days in the same medium at 25–26°C.

3. Behavior to sugars: The fermentative characteristics are found to be after "Lindner's Kleingährmethode."

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	++	++++	+++	—	+	—

4. Fermentation products in "koji"-extract.

	Alcohol.	Esters.	Fuseloil.
A. 7 days at 21–22°C.....	4.49 vol%	————	present.
B. 10 days at 21–22°C.....	4.69 vol%	————	present.
		as ethylacetate.	
C. 20 days at 28°C.....	3.42 vol%	0.2088%	Present.

Of the fine aromas, the flavor of acetacetic ester predominated as in the former varieties.

The following table expresses the formation of organic acid and the amino-acids assimilated in "koji"-extract.

	Amino-acids as glycocoll.				Organic acids as succinic acid.		
	In origi. sol.	After growth.	Assimi- lated.	Coeff. of assimilation.	In orig. sol.	After growth.	Formed.
I. 7 days at 22–25°C.....	0.1391	0.0650	0.074	53.1	0.0344	0.199	0.1650
II. 7 days at 29–30°C.....	0.1270	0.0805	0.0465	26.6	0.0328	0.1204	0.0876
IV. 29 days at 16–19.5°C.....	0.2329	0.152	0.0809	34.7	0.0708	0.1360	0.6520
V. 10 days at 24.5–25.5°C.....	0.2560	0.0576	0.1984	77.0	—	—	—
VI. 10 days at 28°(33°)C.....	0.1590	0.0530	0.1060	66.6	0.0611	0.0980	0.0388
VII. 20 days at 28°C.....	0.2560	0.1760	0.0800	31.0	0.0620	0.1170	0.0550

In this variety, too, the absolute quantities of the amino-acids assimilated are largest in the culture at 24.5–25.5°C. The coefficient of the assimilation of the amino-acids varies from 3.10–77.0, so it shows a close resemblance to var. I.

IV. SACCHAROMYCES SHAOSHING VAR. IV a.

1. Cell and colony: The form of the colonies in "koji"-extract-gelatine (42 days at 15–16.5°C.) is round and waxy with surfacial concentric

rings. These characters resemble the saké-yeast B. 15. The forms of the cell are round or elliptical. The spores are very rarely found even in old cultures in "koji"-extract-gelatine (50 days). The spores found in a cell are small and the maximum number were two. The size of the cells in this culture ("koji"-extract-gelatine) was found to be $7.5 \times 7.5\mu$, $6.2 \times 3.2\mu$ (ellipse), $4.2 \times 4.2\mu$ (the majorities are of this size) $2.5 \times 2.5\mu^3$, so we have already sufficient data to distinguish this variety from the three varieties described. In very old culture (6 months) of "koji"-extract-agar, the round or elliptical spores are found in a few cells. On the gypsum block, the spores are formed after 22-48 hours at 25°C , and in the other media this species behaves quite the same as var. III.

2. Growth: The *streak culture* in "koji"-extract-gelatine (22 days at $15-16^\circ\text{C}$.) has a very similar appearance to var. III, a white and waxy covering. The linear markings on the margin develop more distinctly than in var. III. The same culture in glucose-saké-agar (14 days at $20-21^\circ\text{C}$.) has a smooth surface and is distinguished by this from var. III. The streams on the margin are complicated in this case when compared to var. I and II. A white smooth growth is observable in the surface culture of "koji"-extract-agar (test-tube) (25 days at $22-28^\circ\text{C}$.), and the sediment in condensed water is slightly rose colored, a distinction from var. I and III. The other distinction of this variety from var. III is observed in the surface culture in glucose-saké-agar (test tube) i. e. the covering is white and pasty with a rosy coloration on its thick part. The further distinction of this variety from the var. I. II. III. exists in the same culture at a temperature of $33-34^\circ\text{C}$. (16 days), showing a creamy and slightly rosy colored covering.

Fluid culture: In "koji"-extract (test tube) culture at $18.5-19^\circ\text{C}$. the yeast ring and island are observable after two weeks but, the formation comes sooner, about 6 days at $25-26^\circ\text{C}$.

3. Behavior to sugars.

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	+ ?	++++	++++	---	++++	---

4. Fermentation products. In the culture of "koji"-extract (12°B .) in ERLLENMEYER'S flask the products found were:—

	Alcohol.	Esters.	Fusel oil.
A. 7 days at 21–22°C.....	5.63 vol%	————	present.
B. 10 days ;, ,, , ... 4.69 vol%		————	present.
C. 20 days at 28°C..... .. 3.42 vol%		0.2714	present.

In this fusel oil the presence of iso-propyl-alcohol was highly probable, but the flavor of acetacetic ester was not found at the time of the analysis.

The organic acids formed and the amino-acids assimilated were:—

	Amino acids as glycocoll.				Organic acid as succinic acid.		
	In	After	Assimi-	Coeff. of	In	After	
	origin. sol.	growth.	lated.	assimilation.	origin. sol.	growth.	Formed.
	%	%	%		%	%	%
I. 7 days at 22–25°C.....	0.1391	0.113	0.0278	19.9	0.0344	0.185	0.159
II. 7 days at 29–30°C.....	0.1270	0.0869	0.0401	31.6	0.0328	0.0974	0.0646
IV. 29 days at 16–19.5°C.....	0.2329	0.1750	0.0579	24.4	0.0708	0.1492	0.0784
V. 10 days at 24.5–25.5°C.....	0.2560	0.0661	0.1899	74.0	—	—	—
VI. ,, 28° (33°)C.... .	0.1590	0.098	0.0610	34.3	0.0611	0.1064	0.0472
VII. 20 days at 28°C.....	0.2560	0.1600	0.096	37.6	0.0620	0.1090	0.0470

The largest quantity of amino-acids assimilated absolutely is met with in the culture at 24.5–25.5°C as we have seen in the former cases.

V. SACCHAROMYCES SHAOSHING var. IVb.

1. Cell and colony: The form of the colonies in “koji”-extract-gelatine (42 days at 15–16.5°C) is quite the same as that of var. IVa, analogous to B.15 of saké-yeast. The cell forms are round or elliptic and the size in an old culture of “koji”-extract-gelatine (50 days) measured $7.5 \times 7.5\mu$ (the majority), $3.7 \times 3.7\mu$, $4.2 \times 3.7\mu$ (elliptic). Spores are very rarely found in cells of this culture, but when present, they are found from one to five in a cell. The form of the spore is round or elliptic with 3μ in diameter when round, and 3×2 , 4μ , $4.2 \times 2.5\mu$ when elliptic, but the most common size has a long diameter of 2.5μ . On the gypsum block the spores are found after 19–22 hours at 25°C, and after 5–6 days in diluted “shōyu,” 21–40 hours in pure agar and 24–40 hours in Gorodokowa’s agar. The germination is according to the most common type. In “koji”-extract-gelatine the spores are found in certain cells after 60 days.

2. Growth: The appearance of the *surface culture* of this variety coincides very closely with that of var. IVa, so that we could hardly find any difference between the two cultures. *Fluid culture*: The formation of the yeast ring and the island ensues under quite the same conditions as var. IVa.

3. Behavior to sugars: The fermentative faculties are:—

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	+	++++	+++	—	++++	—

4. Fermentation products: As the products of the fermentation, alcohol, esters and fuseloil were determined.

	Alcohol.	Esters.	Fusel oil.
B. 10 days at 21–22°C.....	4.46 vol%	—	present.
C. 20 days at 28°C.....	4.18 vol%	0.266%	present.

The presence of iso-propyl-alcohol in the fusel oil of this product was highly probable and the pleasant aroma of acetacetic ester predominated among the esters:

The assimilation of amino-acids and the formation of organic acids in the same fluid were found to be:—

Amino acids as glycocoll.				Organic acid as succinic acid.			
	In origin.	After sol. growth.	Assimi- lated	Coeff. of assimilation.	In orig. sol.	After growth.	Formed.
I. 7 days at 22–25°C.....	0.1391	0.0863	0.0428	30.8	0.0344	0.175	0.142
II. 7 days at 29–30°C.....	0.1270	0.0788	0.0482	38.0	0.0324	0.124	0.0915
IV. 29 days at 16–19.5°C.....	0.2329	0.1510	0.0819	35.1	0.0708	0.119	0.0488
V. 10 days at 24.5–25.5°C.....	0.2560	—	—	—	—	—	—
VI. 10 days at 28° (35°) C.....	0.1590	0.0580	0.1010	63.5	0.0311	0.0980	0.0388
VII. 20 days at 28°C.....	0.2560	0.100	0.0960	37.6	0.0620	0.1090	0.0470

VI. SACCHAROMYCES SHAOSHING var. V.

1. Cell and colony: The colonies in “koji”-extract-gelatine (42 days at 15–16°C) are round with a concavity at the central part and with radiated streams on the margin, as we have observed in the gigantic colonies of saké yeast B.7 or B.17. The forms of the cell are round,

elliptic or sausage shape and the size of that cells in the old (50 days) culture in "koji"-extract-gelatine measures $10 \times 4.2\mu$ (sausage), $2.7 \times 2.7\mu$, $6.2 \times 3.7\mu$ (ellipse), $5.6 \times 2.7\mu$ (ellipse). The cell in this culture very seldom contains spores, which have an elliptical form, and chiefly three of them are aggregated together. In "koji"-extract-agar the spores are already observable after 40 days at room temperature. The cells are more polymorphic in this culture i.e. beside round and elliptic, oval, citron or club shaped ones appear. On the gypsum block the generation of the spores²¹ is ascertained after 19 hours and after 40-70 hours in Gorodokowa's agar at 25°C. They germinate according to the most common type of spore germination.

2. Growth: The *surface culture* in "koji"-extract-gelatine (22 days at 15-16°C) has a dirty white smooth surface with the streams in two steps deeply marked on the margin, and this construction of the growth distinguishes this variety from the varieties already described (cf. the plate). The similar surface culture in glucose-saké-agar (14 days at 20-21°C) has a very similar growth to var VI, i.e. the surface of the covering along the streak line expresses basket like folds, which develop more coarsely than that of var. VI. The surface culture in "koji"-extract agar (25 days at 22-28°C) (test tube) develops a smooth yellow covering with a *brown deposit* in condensed water, and by this character we can distinguish the varieties already described. On the other hand, in glucose-saké-agar (11 days at 21-22°C) the basket like folded covering colors yellowish rosy red, and by this character may be distinguished from the varieties already described with the exception of var. III. *Fluid culture*: In "koji"-extract (test tube) a white yeast ring ensues after two weeks culture at 18.5-19°C and commences to form after 6 days at 24-25°C.

4. Behavior to sugars: The fermenting property differs somewhat from the varieties described, thus:—

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	+	++++	++++	—	++	+

The fermenting character of this variety for dextrin, though in a slight

21. The duration of the spore formation in the other media coincides with that of var. IV b.

degree, is an interesting fact, as we have very few varieties of yeast of this kind.

4. Fermentation products: In “koji”-extract culture the products found were :—

	Alcohol.	Esters.	Fusel oil.
A. 7 days at 21-22°C.....	5.55 vol%	————	present.
B. 10 days at 21-22°C.....	4.78 vol%	————	present.
C. 20 days at 28°C,.....	4.88 vol%	0.27%	present.

The pleasant aroma of acetacetic ester was not noticeable in the culture, but the accompaniment of isopropyl-alcohol in fuseloil was highly probable.

As regards to amino-acids and organic acids in “koji”-extract the following may be mentioned.

Amino-acids as glycocoll.					Organic acids as succinic acid.			
		In	After	Assimi-	Coeff. of	In	After	Formed.
		orig. sol.	growth	lated.	assimilation.	orig.sol.	growth.	
		%	%	%		%	%	
I.	7 days at 22-25°C.....	0.1391	0.0712	0.0679	48.8	0.0344	0.171	0.138
II.	7 days at 29-30°C.....	0.1270	0.0745	0.0525	41.3	0.0328	0.0875	0.0547
IV.	29 days at 16-19.5°C.....	0.2329	0.1310	0.1019	43.7	0.0708	0.1408	0.0700
V.	10 days at 24.5-25.5°C....	0.2560	—	—	—	—	—	—
VI.	10 days at 28° (33°) C.....	0.1590	0.0530	0.1060	66.6	0.0611	0.0932	0.0340
VII.	20 days at 28°C	0.2560	0.1640	0.0920	36.0	0.0620	0.0890	0.0270

VII. SACCHAROMYCES SHAOSHING var. VI.

1. Cell and colony: The colonies in “koji”-extract-gelatine (42 days at 15-16.5° C) are round in form with elevated surface at the central part, around which exist many radiated linear markings and at the margin there are small streams. These characteristics remind us of the gigantic colony of saké yeast B. 24.

The sausage formed, round or elliptic cells are found in the old culture (50 days) of this variety in “koji”-extract-gelatine, and measure $12.5 \times 2\mu$, $3.7 \times 5\mu$, $3.7 \times 2.5\mu$, $5 \times 3.7\mu$. Spores are not found in this culture but they are decidedly perceived in the culture in “koji”-extract-agar after 6 months. Two or three elliptic spores are found in a cell. On the gypsum block the

spores are generated after 19–22 hours at 25°C, 70–90 hours in pure agar, 70–94 hours in Gorodokowa's agar, 7 days in "koji"-extract-agar and 9–12 days in diluted "shōyu". They germinate after the most common type.

2. Growth: The *surface culture* in "koji"-extract-gelatine (22 days at 15–16°C) has a yellowish white waxy consistency with a smooth surface at the central part, but on the margin there appear many fine striations. At this stage the gelatine has commenced to dissolve already along the streak lines. The similar culture in glucose-saké-agar (14 days at 20–21°C) gave a covering very much like variety V, basket like folds, with the difference, however, that they develop more finely and deeply in this case than var. V. The wider streams on the margin of the covering are a character which must not be overlooked to distinguish this variety from var. V. The *streak culture* in "koji"-extract-agar (test tube; 25 days at 22–28°C) makes a white and smooth covering with weak streams on the margin, moreover, the growth in the thicker part of the medium and the deposit formed in condensed water are colored somewhat rosy-red. Such coloration of the growth is entirely absent in this medium in all varieties already described, except var. III, which, however, gives a lighter colored growth and wider markings than this variety on the margin.

Fluid culture: In "koji"-extract (test tube) the yeast ring appears after two weeks at 18.5–19°C, and after 6 days at 24–25°C.

3. Behavior to sugars: The fermenting property is shown:—

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	++	++++	+++	—	?	—

4. Fermentation products: The products in "koji"-extract are:—

	Alcohol.	Exters.	Fusel oil.
A. 7 days at 21–22°C.....	4.78 vol%	————	present.
B. 10 days „ „	————	————	present.
C. 20 days at 28°C.....	3.42 vol%	0.2607%	present.

Of the agreeable aroma, the flavor of acetacetic ester predominated and the presence of isopropyl-alcohol in fusel oil as an accessory ingredient was highly probable.

The change in the amino-acids and the organic acids is shown below:—

		Amino-acids as glycocoll.				Organic acids as succinic acid.		
		In orig. sol.	After growth.	Assimi- lated,	Coeff. of assimilation.	In orig.sol.	After growth.	Formed.
		%	%	%		%	%	%
I.	7 days at 22-25°C.....	0.1391	0.0412	0.0979	70.3	0.0344	0.1420	0.108
II.	7 days at 29-30°C.....	0.1270	0.057	0.0763	52.2	0.0328	0.1448	0.1156
IV.	29 days at 16-19.5°C.	0.2329	0.1310	0.1019	43.7	0.0708	0.1220	0.0512
V.	10 days at 24.5-25.5°C.....	0.2560	0.0492	0.208	80.0	—	—	—
VI.	10 days at 28° (33°) C.....	0.1590	0.0530	0.1060	66.6	0.0611	0.148	0.0556
VII.	20 days at 2 °C.....	0.1560	0.1720	0.0840	32.8	0.0620	0.09	0.0270

The assimilation of the amino-acids is most conspicuous at a temperature of 24.5-25.5°C, and in comparing the coefficient of the assimilation of the acid to saké yeast, this variety behaves as the variety which gives the maximum coefficient.

VIII. SACCHAROMYCES SHAOSHING var. VII.

1. Cell and colony: The round waxy colony is found in "koji"-extract-gelatine (15-16.5°C)(42 days) and the margin of it consists of light marked streams, as we have observed in the gigantic colony of saké yeast A5. Generally the forms of the cell are elliptic, club-like and sausage shape. The sizes in the old culture (50 days) in "koji"-extract-gelatine measure $5 \times 3.7\mu$, $7.5 \times 2.5\mu$, $10 \times 2.5\mu$, $5.2 \times 4.2\mu$, $5 \times 4.2\mu$. Many of the cells already contain one to three spores, round or elliptic, at this stage of the culture. The size of the spores is variable but commonly measures $2.5 \times 2.5\mu$ or $2.5 \times 1.3\mu$. The spore carrying cells are found in the culture of "koji"-extract-agar after 40 days at room temperature, so we can very easily find these cells after 6 months culture of the medium. On the gypsum block the spore formation ensues after 19 hours at 25°C, after 14 days in "koji"-extract-agar, after 9-12 days in diluted "shōyu."

2. Growth: The *surface culture* in "koji"-extract-gelatine (in PETRI's dish) (22 days at 15-16°C) develops as a dirty white covering, as var. V, but conveys very fine linear marking on the margin. The similar culture in glucose-saké-agar (14 days at 20-21°C) makes a growth as observed in var. V and VI, but differs from them in having a less folded surface. The *streak*

culture in “koji”-extract-agar (25 days at 22–28°C) (test tube) appears as a smooth yellowish-white covering with the linear markings on the margin. The deposit in condensed water in this culture is colored rosy-red. The like culture in glucose-saké-agar (11 days at 21–22°C) appears as a rosy-red colored covering with a few folds but with a graular appearance at the thick part of the medium. The growth at the thin part of the medium is of a yellow tint. - *Fluid culture*: In “koji”-extract a yeast ring begins to form after two weeks at 18·5–19°C, after 6 days at 24–25°C.

3. Behavior to sugars: The fermentative property of this variety for sugars is shown as:—

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	+	+++	++++	—	+++	—

4. The products of fermentation: The contents of alcohol in “koji”-extract after fermentation are:—

	Alcohol.	Fusel oil.
A. 7 days at 21–22°C.....	4·71 vol%	present.
B. 10 days at 21–22°C	4·34 vol%	present.
C. 20 days at 28°C.....	2·02 vol%	present.

A trace of the flavor of acetacetic acid was found in the distillate.

The change of amino-acids and the organic acids found to be:—

Amino-acids as glycocoll.					Organic acids as succinic acid %.		
	In	After	Assimi-	Coeff. of	In	After	
	orig. sol.	growth.	lated.	assimilation.	orig. sol.	growth.	Formed.
I. 7 days at 22–25°C	0.1191	0.0621	0.0770	54.7	0.0344	0.382	0.346
II. 7 days at 22–35°C.....	0.1270	0.0868	0.0402	31.6	0.0328	0.088	0.0550
IV. 29 days at 16–19.5°C.....	0.2329	0.1310	0.1019	52.3	0.0708	0.124	0.0536
V. 10 days at 24.5–25.5°C.....	0.2560	0.0678	0.1882	73.0	—	—	—
VI. 10 days at 28° (33°) C.....	0.1590	0.0670	0.092	57.7	0.0611	0.1008	0.416
VII. 20 days at 25°C.....	0.2560	0.176	0.080	31.0	0.0620	0.708	0.646

The optimum temperature for the assimilation of the amino-acids lies near at 24–25°C.

Summary of Part I.

Saccharomyces shaoshing, characteristic of the "shaoshing-chŭ"-mash, is a new species of yeast analogous to saké-yeast, but differing especially by a very quick generation of yeast ring, i.e. in 6 days at 24–25°C, by the duration of the spore formation, and further by the formation of the characteristic rosy-red coloration in the growth of glucose-saké-agar or even in "koji"-extract-gelatine in some varieties.* All the varieties described, eight in all, ferment glucose, maltose, saccharose, raffinose and sparingly galactose, but not lactose and dextrine. One variety, var. V, *ferments dextrine*, though in a very slight degree, which must be treated as a very interesting fact. The pleasant aroma, chiefly acetacetic ester, developed by the species, is found conspicuously in "koji"-extract culture in ERLÉNMEYER'S flask. Such flavor is noticed by the other yeast, i.e. saké yeast, only in the culture of PASTEUR'S flask with an exuberant supply of air. The production of alcohol varies in accordance with the varieties, i.e. in round numbers 4–5.5 vol%. The coefficient of the assimilation of the amino-acids varies too, according to the varieties, the maximum reaching 80 at 24.5–25.5°C. The conditions for the spore formation of the varieties are summarized below (after MASUNAGA).

	On At 25°C. gypsum block.	In diluted "shoyu"	In "koji"-extract agar.	In pure agar.	In Gorodokowa's agar.
Var. I.	19h ^s	4–5 days	14 days	21h ^s	24–40h ^s
Var. II.	19h ^s	4–5 days	6–7 days	21h ^s	24–40h ^s
Var. III.	19–20h ^s	9–12 days	14 days	—	—
Var. IV. a.	22–40h ^s	9–12 days	14 days	—	—
Var. IV. b.	19–22h ^s	5–6 days	14 days	21–40h ^s	24–40h ^s
Var. V.	19h ^s	5–6 days	14 days	—	40–70h ^s
Var. VI.	19–22h ^s	9–12 days	7 days	70–90h ^s	70–94h ^s
Var. VII.	19h ^s	9–12 days	14 days	—	—

Part II. Zygosaccharomyces.

I. ZYGOSACCHAROMYCES SHAOSHING var. I. NOV. SP.

1. Cell and colony: The colonies formed in "koji"-extract-gelatine

(42 days at 15–16.5°C) are round and elevated with a smooth surface, and the margin is constructed of several streams. The form of the cell is round or elliptic and the size in the old culture (50 days at 15–16.5°C) measure $2.5 \times 2.5\mu$, $5 \times 5\mu$, $6.2 \times 8.7\mu$. The spore formation was not complete at this stage of the culture, but certain cells contained a fine round spore-like substance, which stained slightly blue by the solution of iodine-K-iodide. These spore-like granules are large in size when only one of them occurs in a cell. A further noticeable phenomenon is that these spore-like granules occur constantly in one of the two connected cells, and between two cells a canal is observable very clearly when stained by iodine-K-iodide solution. These connected cells generally are unequal in size, i.e. a small one and a large one, as if they were micro- and macrogametes. The size of the microgamete measure $3.5 \times 3.5\mu$, and that of the macrogamete $5 \times 5\mu$ or $7.5 \times 7.5\mu$. The spore-like substances are found abundantly in the large cell; however, the germination of these substances at this stage has not yet been observed. The cells in the culture of “koji”-extract-agar after 5 months contain one or three round or elliptical spores, which stain well by ZIEHL’s carbol fuchsin solution but the greater part of it is decolorized by HCl-alcohol, leaving a small round point stained at the central part, as we have observed in the case of *Zygosaccharomyces* of “shoyu”-mash. *These spores germinate very easily as shown in the plate.* On the gypsum block the spore-making experiment failed after 2 days at 25°C.

2. Growth: The *surface growth* in “koji”-extract gelatine (PETRI’S dish) (22 days at 15–16°C) appears as a white waxy covering with fine markings on the margin. The similar culture in glucose-saké-agar (14 days at 20–21°C) gives a smooth and elevated covering of which the marginal part is constituted of wide streams. The *streak culture* in “koji”-extract-agar (test tube) (25 days at 22–28°C) develops as a greyish white covering, and the deposit formed in condensed water is colored brown. On the other hand, in glucose-saké-agar (11 days, 20–22°C) the growth appears as a white waxy covering with linear markings on the margin, and the growth in the thick medium is colored rosy-red. The coloration of the covering is not observable when the temperature of the culture is kept at

33–34°C (16 days) in the same culture, and the covering remains white and smooth. White and folded coverings are obtained in the culture of saké-agar streak culture. (18 days at 20–21°C).

Fluid culture: The yeast-ring is found after two weeks (18.5–19°C) in “koji”-extract and islands begin to form at the same time.

3. Behavior to sugars: The fermenting property for sugar is found:—

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
++++	++	++++	++++	—	++++	+

4. Fermentation products: In “koji”-extract the fermentation products found were:—

	Alcohol.	Esters.	Fusel oil.
A. 7 days at 21–22°C.....	3.98	————	present.
B. 10 days „ „	4.54	————	present.
C. 20 days 28°C.....	4.88	0.269	present.

In the pleasant aroma of the fluid, acetacetic esters predominated and the presence of isopropyl-alcohol as accessory ingredient of the fusel oil was highly probable.

The fate of the amino-acids and organic acids in “koji”-extract during fermentation may be traced from the following data:—

Amino-acids as glycocoll.				Organic acids as succinic acid.			
	In	After	Aisimi- lated.	Coeff. of assimilation.	In	After	Formed.
	orig. sol. %	growth. %			orig. sol. %	growth. %	
I. 7 days at 22–25°C.....	0.1391	0.0836	0.0550	39.9	0.0344	0.161	0.127
II. 7 days at 29–30°C.....	0.1270	0.01054	0.0216	17.0	0.0328	0.124	0.0915
IV. 29 days at 16–19.5°C.....	0.2329	0.1990	0.0339	14.5	0.0708	0.147	0.0764
V. 10 days at 24.5–25.5°C....	0.2560	0.061	0.1950	76.0	—	—	—
VI. 10 days at 25° (33°) C.....	0.1590	0.0690	0.0900	56.0	0.0611	0.0849	0.0257
VII. 20 days at 25°C.....	0.2500	0.1520	0.1040	46.0	0.0620	0.103	0.0410

The optimum temperature for the assimilation of the amino-acids seems to lie between 24.5–25.5°C. The quantity of organic acids formed is inversely proportional to their quantity originally present in “koji”-extract and indifferent to the temperature of the culture.

II. ZYGOSACCHAROMYCES SHAOSHING var. II. NOV. SPEC.

1. Cell and colony: The colony in "koji"-extract-gelatine (42 days at 15–16.5°C) has a very similar form to var. I, differing in this variety by having streams of two steps on the margin. The round or elliptic cells are found in common, and the size in the old culture (50 days) in "koji"-extract-gelatine measures $5 \times 5\mu$, $2.5 \times 2.5\mu$, $5 \times 2.5\mu$. The spores were not perceived at this stage, but two gametes conjugated already, holding the granular substances in each cell. After 6 months culture in "koji"-extract-agar there are found many cells containing one round or elliptic spore, which stains well by ZIEHL's carbol-fuchsin solution, but peculiar to this variety is, that the stained spore decolorizes relatively easily by HCl-alcohol, leaving only a small part stained. The conjugated cells are found, too, in diluted "shoyu"-culture as observed in "shoyu"-yeast, but they are not met with on the gypsum block at 25°C even after 48 hours.

2. Growth: The *surface culture* in "koji"-extract-gelatine (22 days at 15–16°C) appears very similar to var. I, but differs in giving finer linear markings than the latter on the margin (cf. the plate). The similar culture in glucose-saké-agar forms a covering which holds basket-like folds at both ends of the streak line. The *streak culture* in "koji"-extract-agar (25 days at 22–23°C) appears as a dirty white smooth covering, giving a brown deposit in condensed water. On the other hand, a *waxy* and slightly *rosy-red colored* growth is observed, *granulated at the central part*, with many linear markings on the margin. Such growth of the culture must not be overlooked as a distinguishing property from variety I. The rosy-red coloration of the covering comes after 16 days at 33–34°C. In saké-agar (18 days at 20–21°C) a coarse granulated mesenteric folded covering develops, which is colored very slightly rosy-red at the thick part of the culture medium. *Fluid culture*: In "koji"-extract a yeast ring makes its appearance after two weeks at 18.5–19°C, or after 6 days at 24–25°C.

3. Behavior to sugars: The result of the small fermenting method after LINDNER is shown:—

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrin.
+++	—	+++	++++	—	++++	—

4. Fermentation products: The products of the fermentation in “koji ”-extract were :—

	Alcohol.	Ester.	Fusel oil.
A. 7 days at 21–22°C.....	————	————	————
B. 10 days at 21–22°C.....	4.31 vol%	————	Present.
C. 20 days at 28°C.....	3.42 vol%	0.263%	Present.

The flavor of acetacetic ester in the fluid was perceivable, though not intense.

The change of the amino-acids and the organic acids of “koji ”-extract after fermentation were found to be :—

Amino-acids as glycocoll.					Organic acids as succinic acid.			
		In	After	Assimi-	Coeff. of	In	After	Formed.
		orig. sol.	growth.	lated.	assimilation.	orig. sol.	growth.	
		%	%	%		%	%	%
I.	7 days at 22-25°C.....	0.1391	0.0835	0.0556	39.9	0.0344	0.194	0.160
II.	7 days at 29-30°C.....	0.1270	0.0634	0.0616	48.5	0.0328	0.098	0.066
IV.	29 days at 16-19.5°C.....	0.2329	0.1350	0.0970	42.0	0.0708	0.110	0.080
V.	10 days at 24.5-25.5°C.....	0.2560	—	—	—	—	—	—
VI.	10 days at 28° (33°) C.....	0.1590	0.0630	0.0960	60.3	0.0611	0.090	0.040
VII.	20 days at 28°C.....	0.2560	0.1760	0.0800	31.00	0.0620	0.109	0.047

III. ZYGOSACCHAROMYCES SHAOSHING var. III. NOV. SPEC.

1. Cell and colony: In “koji ”-extract-gelatine, this variety develops as a round colony, flat at the central part surrounded by radial markings. The cell forms are round, oval or elliptic, and the size in old cultures (50 days) of “koji ”-extract-gelatine is $7.5 \times 7.5 \mu$, $4.2 \times 4.2 \mu$, $2.5 \times 2.5 \mu$, $3 \times 2.5 \mu$. Some of the cells contained spores at this stage, differing from the varieties I. and II. of *Zygosaccharomyces* described. Two or three round or elliptic spores are found developed in a cell. The base of the spore, round in shape, is found in the macrogamete. In “koji ”-extract-agar the spores are generated after 60 days culture, and the cells containing them increase after further cultivation (6 months). They stain well by ZIEHL’s carbol fuchsin solution and the nondecolorizing part of the stained spore by HCl-alcohol, peculiar to note, lies on the short axis of the elliptic spore in the shape of the crescent moon. The germination is possible by

the spores contained in the isolated cell i.e., the matured spores (cf. the plate). On the gypsum block the generation of the spores was not possible even after two days at 25°, but they were perceived after 21–40 hours in pure agar and after 24–40 hours in Gorodokowa's agar.

2. Growth: A white waxy smooth covering was obtained in "koji"-extract-gelatine as a *surface culture* (PETRI'S dish, 22 days at 15-16°C). The surface culture in glucose-saké-agar (14 days at 20-21°C) appears very similar to var. I. The *streak culture* in "koji"-extract-agar (25 days at 22-23°C) (test tube) gave a light yellowish dirty white covering with smooth surface, and the sediment in condensed water colored very lightly rosy-reddish brown. A smooth and pasty rosy-red colored covering was obtained in the similar culture of glucose-saké-agar and the coloration develops more intensely at 33-34°C by the same medium (16 days). The growth in saké-agar (18 days at 20-21°C) has a smooth surface and is colored rosy-red at the thick part of the medium. *Fluid culture*: The conditions for the generation of the yeast ring and island are quite the same as in var. I.

3. Behavior to sugars: According to Lindner's small fermenting method the result of the fermenting property for the sugar was:—

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrine.
++++	++	++	+++	—	++	—

4. Fermentation products in "koji"-extract.

	Alcohol.	Esters.	Fusel oil.
A. 7 days at 21–22°C.....	5.49 vol%	————	present.
B. 10 days at 21–22°C.....	2.43 vol%	————	present.
C. 20 days at 28°C.	3.42 vol%	0.259%	present.

In the pleasant aroma, the flavor of acetacetic ester was ascertained to be copiously present, and in the distillate the presence of the secondary butyl-alcohol as an accessory ingredient of the fusel oil is highly probable.

The fate of the amino-acids and the organic acids in "koji"-extract may easily be traced from the data below.

Amino-acids as glycocoll.					Organic acids as succinic acid.			
		In	After	Assimi-	Coeffi. of	In	After	
		orig. sol.	growth	lated.	assimilation.	orig. sol.	growth.	Formed.
		%	%	%		%	%	%
I.	7 days at 22-25°C.....	0.1391	0.0863	0.0428	51.7	0.0344	0.161	0.1270
II.	7 days at 29-30°C.....	0.1270	0.0783	0.0432	26.8	0.0323	0.099	0.0776
IV.	29 days at 16-19.5°C.	0.2329	0.1240	0.1089	46.9	0.0708	0.135	0.0648
V.	10 days at 24.5-25.5°C	0.2560	0.0729	0.1830	71.0	—	—	—
VI.	10 days at 28 (33°) C.	0.1590	0.04590	0.1131	70.9	0.0611	0.088	0.0496
VII.	20 days at 28°C.....	0.2560	0.1760	0.0800	31.0	0.0320	0.109	0.0470

The maximum assimilation of the amino-acids was observed at temperature of 24.5-25.5°C.

IV. ZYGOSACCHAROMYCES SHAOSHING var. IV. NOV. SPEC.

1. Cell and colony: The colony in “koji”-extract-gelatine is round as described in the case of var. VII. of *Saccharomyces shaoshing*. The form of the cell is round, oval or elliptic, and the size of the cell in “koji”-extract-gelatine (50 days culture) is $6.2 \times 3.7\mu$, $7.5 \times 5\mu$, $7.5 \times 7.5\mu$, $8.7 \times 8.7\mu$, $3.7 \times 2.5\mu$. In this culture, conjugated cells and some deformed cells were found, the former contained a spore-like substance at its base. Moreover, certain isolated cells were filled with 2 or 4 spores by this culture, while in the culture of “koji”-extract-agar the spores were perceived after 55 days. On the gypsum block the spore culture failed to form after 48 hours at 25°C.

2. Growth: The *surface culture* in “koji”-extract-gelatine (22 days at 15-16.5°C) appears as a waxy covering with coarse and marked streams on the margin, differing from all the varieties described in this paper. A white waxy covering makes its appearance in “koji”-extract-agar (25 days at 22-28°C) (test tube) surface culture, while a light yellow colour ensues after 16 days at 21-23°C. In glucose-saké-agar (11 days at 20-22°C) a basket-like folded covering with rosy-red coloration is found, which in the same medium at 33-34°C develops as a creamy rosy red colored covering with mesenteric folds, and may be distinguished from other *Zygosaccharomyces* varieties in this respect. In saké-agar at 20-21°C (18 days) a light

rosy red colored granular growth is found. *Fluid culture*: In "koji"-extract the yeast ring with yellow coloration is found after two weeks at 18.5–19°C and after 6 days at 24–25°C.

3. Behavior to sugars: In fermenting property this variety differs from var. III. by showing a better fermentation for raffinose and from var. II. by fermenting galactose.

Glucose.	Galactose.	Maltose.	Saccharose.	Lactose.	Raffinose.	Dextrine.
++++	++	+++	++++	—	++++	—

4. Fermentation products: The products of the fermentation in "koji"-extract found were:—

	Alcohol.	Ester.	Fusel oil.
A. 7 days at 21–22°C.....	4.44 vol%	————	present.
B. 10 days at 21–22°C.....	4.93 vol%	————	present.
C. 20 days at 28°C.	4.88 vol%	0.264%	present.

The change of the amino-acids and the organic acids in "koji"-extract after fermentation is shown below:—

	Amino-acids as glycocoll.				Organic acids as succinic acid.		
	In	After	Assimi-	Coeff. of	In	After	
	orig. sol.	growth.	ated.	assimilation.	orig. sol.	growth.	Formed.
	%	%	%		%	%	%
I. 7 days at 22–25°C.	0.1331	0.1016	0.0375	26.8	0.0344	0.156	0.122
II. 7 days at 29–30°C.	0.1270	0.152	+0.0259	—	0.0328	0.0996	0.0668
IV. 29 days at 16–19.5°C.	0.2329	0.143	0.0899	38.5	0.0708	0.1264	0.0556
V. 10 days at 24.5–25.5°C.....	0.2560	0.061	0.1950	76.0	—	—	—
VI. 10 days at 28' (33°) C.....	0.1590	0.064	0.095	59.7	0.0611	0.086	0.0263
VII. 20 days at 28°C.....	0.2560	0.176	0.080	31.0	0.0620	0.117	0.0550

The assimilation of the amino-acid was limited below its formation in the culture at 29–30°C, so there was observed an increase instead of a decrease of it. The maximum assimilation of the amino-acids was found in this variety also at 24.5–25.5°C.

Summary of Part II.

Four new varieties of *Zygosaccharomyces shaoshing* have been found, though there remains a minute point not cleared up. The peculiar form of the cells or the conjugation and the sporulation therein is sufficient to

define them as *Zygosaccharomyces*. The germination, however, is observed only by the spores contained in the isolated cell and not by the cells conjugated. So that the conjugation of the cells is a preliminary step of the sporulation. The spore stains well by ZIEHL's carbol-fuchsin solution, but a small part of it remains not decolorized by HCl-alcohol, as we have observed in our *Zygosaccharomyces* of "shoyu"-mash. The incapability of the spore formation on the gypsum block is observed in this as we have mentioned in "shoyu"-*Zygosaccharomyces*, but the formation by this species is perceived in the common medium such as "koji"-extract-agar or -gelatine, which is unsuitable for spore formation in the latter case. In the diluted "shoyu" and Gorodokowa's agar the spore formation is not met with, except in var. III. of this species.

Among the other distinctions from the "shoyu"-*Zygosaccharomyces* may be mentioned the rosy-red coloration of the growth and the faculty to ferment raffinose²² and galactose.²³ *Zygosacch. salsa* and *Zygosacch. japonicus* Saito are film forming species, so they may be distinguished safely from the present varieties. The other species hitherto described in the literature are distinguished from the writer's species thus:—

Species.	Is distinguished
<i>Zygosacch. Barkeri</i> Saccardow. ²⁴	by not fermenting maltose, raffinose.
<i>Zygosacch. priorianus</i> Klöcker. ²⁵	by not fermenting saccharose, raffinose and fermenting very feebly or in a doubtful degree maltose and galactose.
<i>Zygosacch. lactis</i> α Dombrowski.	by fermenting lactose.
<i>Zygosacch. javanicus</i> Kruyff. ²⁶	by not fermenting maltose, and by fermenting galactose from var. II, by not fermenting dextrine from var. I.
<i>Zygosacch. fusoriens</i> Saito. ²⁷	by not fermenting raffinose and saccharose, and by fermenting dextrine.
<i>Zygosaccharomyces</i> from cacao. ²⁸	by not fermenting saccharose, and fermenting <i>energetically</i> dextrine.

22. *Zygosacch. major* do not ferment raffinose.

23. *Zygosacch. major* and *soja* could not ferment galactose.

24. LAFAR. Handbuch d. Tech. Mykol. IV. S. 182.

25. LAFAR. Handbuch d. Tech. Mykol. IV. S. 182. u. Woch. f. Brau. 1911. No. 6. LINDNER's article.

26. Centlb. f. Bak. II. d. XXI. 619.

27. Woch. f. Brauerei. 1911. Nr. 6. LINDNER's article.

28. Woch. f. Brauerei. 1911. Nr. 6. LINDNER's article.

Part III. Mycoderma, Mould Fungus and Bacteria.

Some varieties of *Willia anomala* were found in "Chŭya." Their properties will be reported in the next journal.

Among the mould fungi, *Penicillium glaucum*, a variety of *Mucor* and *Chaëta* were found, but they may be regarded of minor importance.

In regard to the species of the bacteria in "shaoshing-chŭ"-mash, two species of *butyric acid bacillus* and one species of *lactic bacillus* were isolated. The details will be reported in a later number of this journal, but it is very reasonable to believe that they play an important role in the fermentation of "shaoshing-chŭ"-mash.

Addendum.

Recently, the author has found some varieties of *Willia anomala* in the sediment of "shaoshing-chŭ." Their details will be published in the further journal.

EXPLANATION OF PLATES.

PLATE IX.

The colonies in "koji"-extract gelatine.

I.	<i>Saccharomyces shaoshing.</i>	var. I.
II. a.	"	var. II.
II. b.	"	var. III.
III.	"	var. IV. a.
III. c.	"	var. IV. b.
IV. a.	"	var. V.
K. s.	"	var. VI.
S.	"	var. VII.
II. c.	<i>Zygosaccharomyces shaoshing.</i>	var. I.
II. d.	"	var. II.
III. a.	"	var. III.
M.	"	var. IV.

PLATES X AND XI.

Photographic representations of the cell of each variety from old culture in "koji"-extract agar.

PLATE XII.

Successive stages of the germinated spore in hanging drop. Arabic numerals on each figure show the time of observation in hours.

Fig. 1.	<i>Saccharomyces shaoshing.</i>	var. I,	spores were taken from
Fig. 2.	„	var. II.,	gypsum block after 10 days culture.
Fig. 3.	„	var. III.,	gypsum block after 2 days culture.
Fig. 4.	„	var. IVa.,	“ koji ”-extract-agar after 6 months.
Fig. 5.	„	var. IVb.,	gypsum block after 3 days culture.
Fig. 6.	„	var. V.,	„ „ „ 2 days culture.
Fig. 7.	„	var. VI.,	„ „ „ 3 days culture.
Fig. 8.	„	var. VII.,	„ „ „ 4 days culture.
Fig. 9.	<i>Zygosaccharomyces shaoshing.</i>	var. III.,	diluted “ shōyu ” after 14 days.

PLATE XIII.

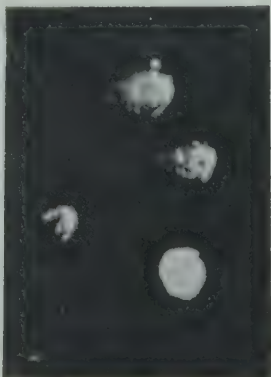
The surface culture in glucose-saké-agar (PETRI's dish).

PLATE XIV.

The surface culture in "koji"-extract-gelatine (PETRI's dish).



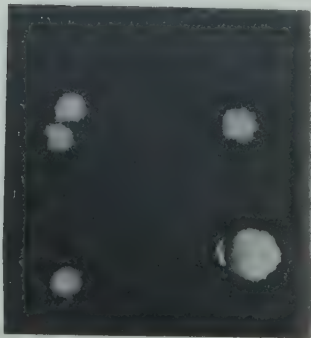
I.
(10/II-17/IV)



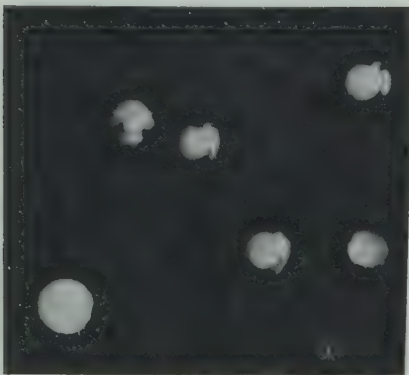
II. a
(10/II-17/IV)



II. b
(10/II-17/IV)



II. c
(24/II-17/IV)



II. d
(10/II-17/IV)



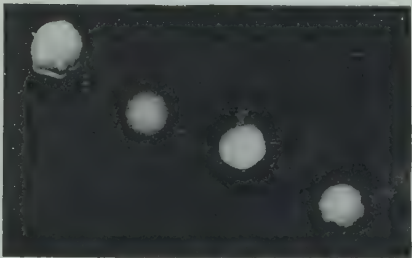
III. a
(10/II-17/IV)



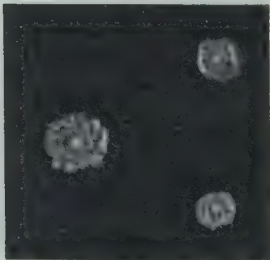
III. b
(10/II-17/IV)



IV. a
(10/II-17/IV)



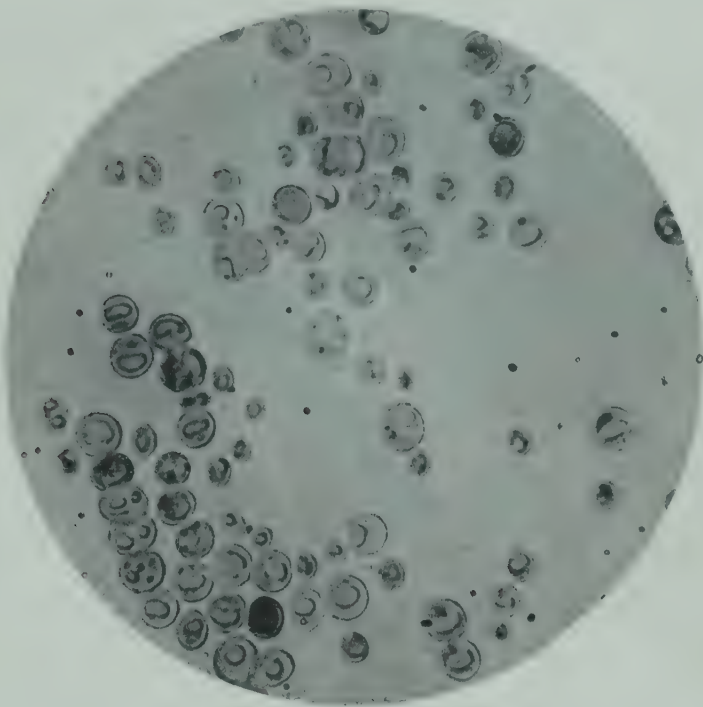
S
(11/II-17/IV)



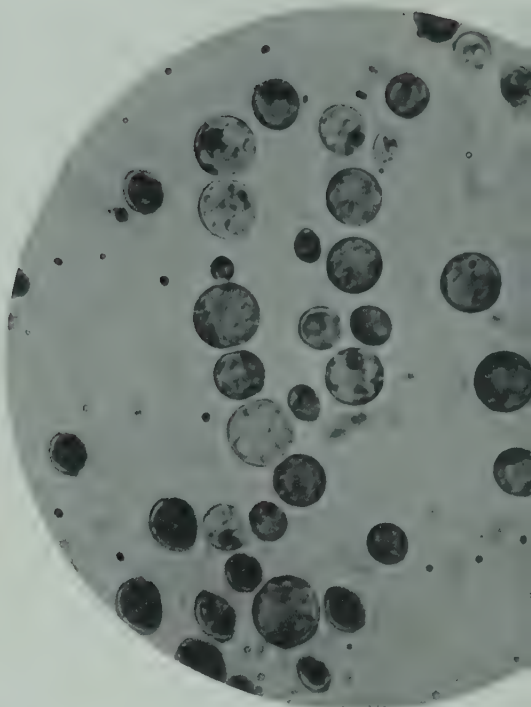
K. s
(10/II-17/IV)



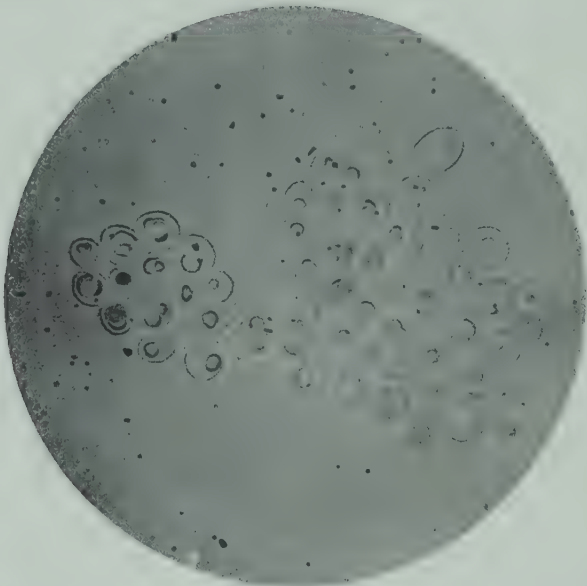
M
(24/III-28/IV)



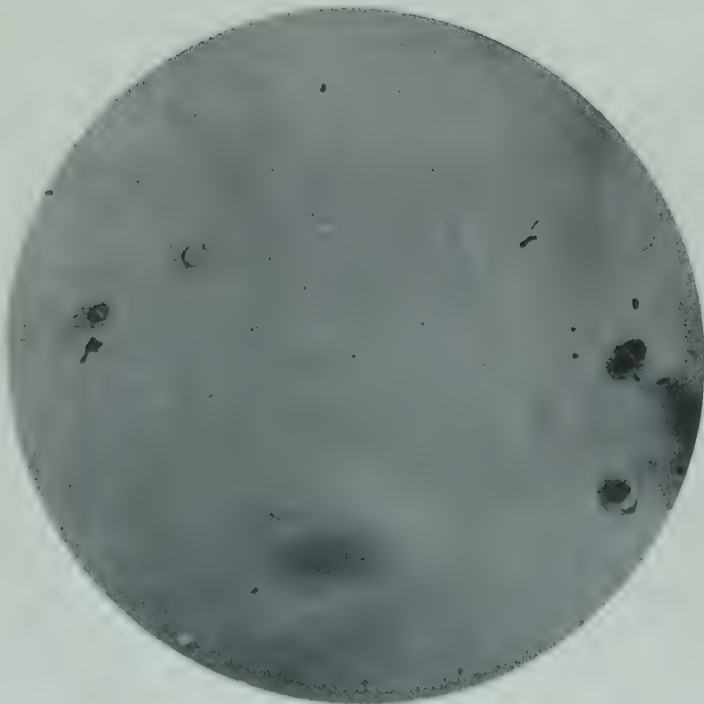
III. b



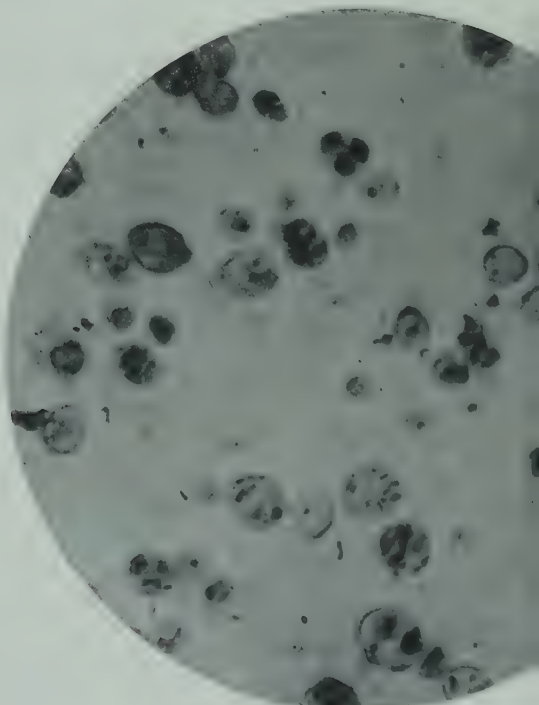
II. c
(8/III)



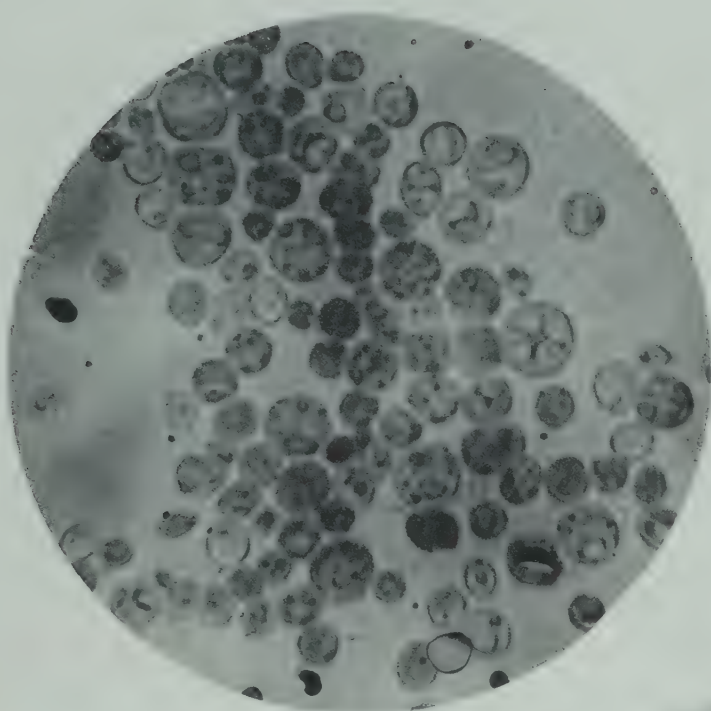
I



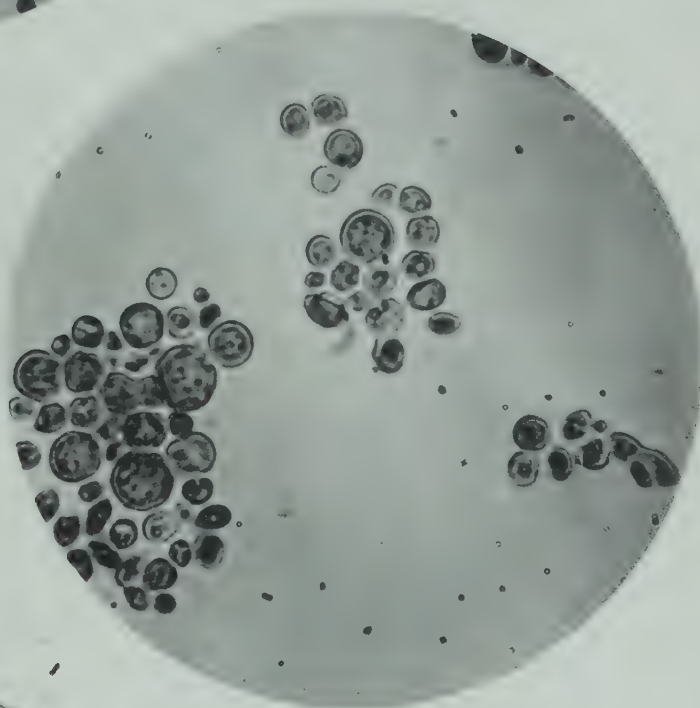
S
(28/X)



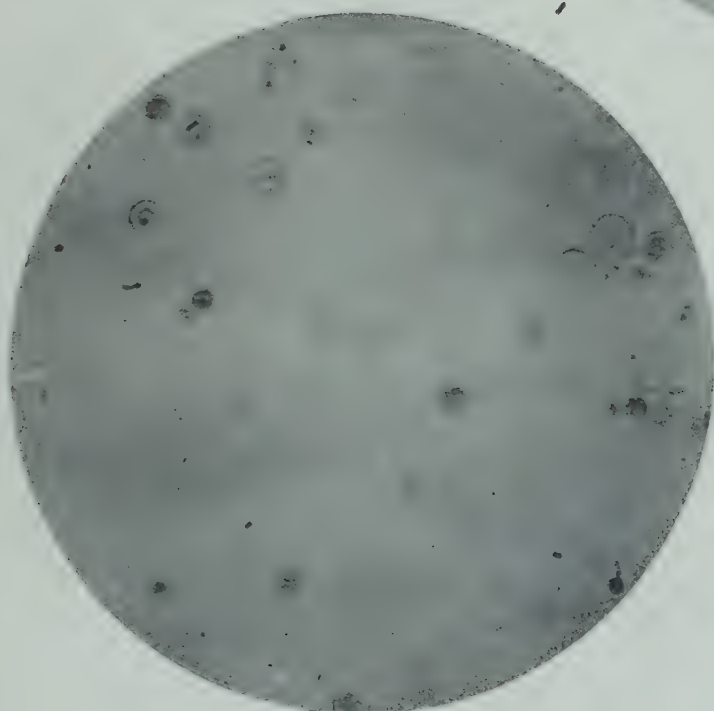
II. a
(4/XI)



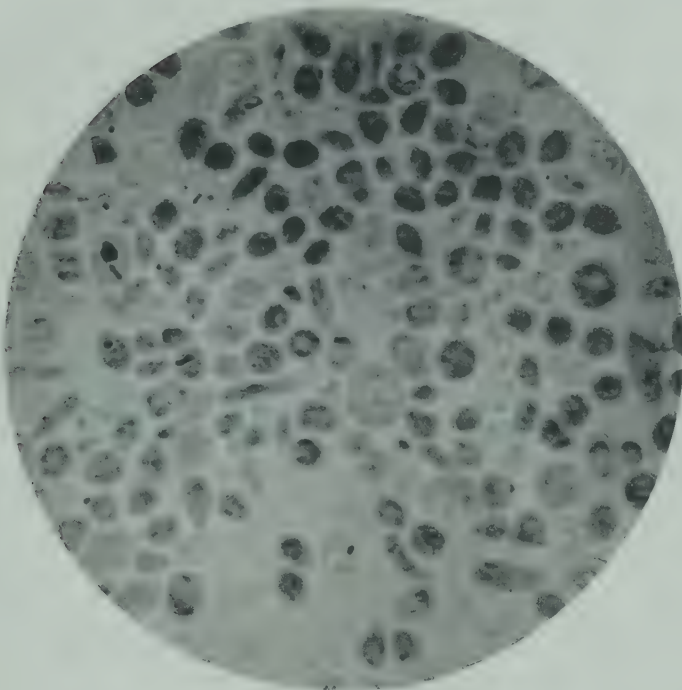
I
(9/III)



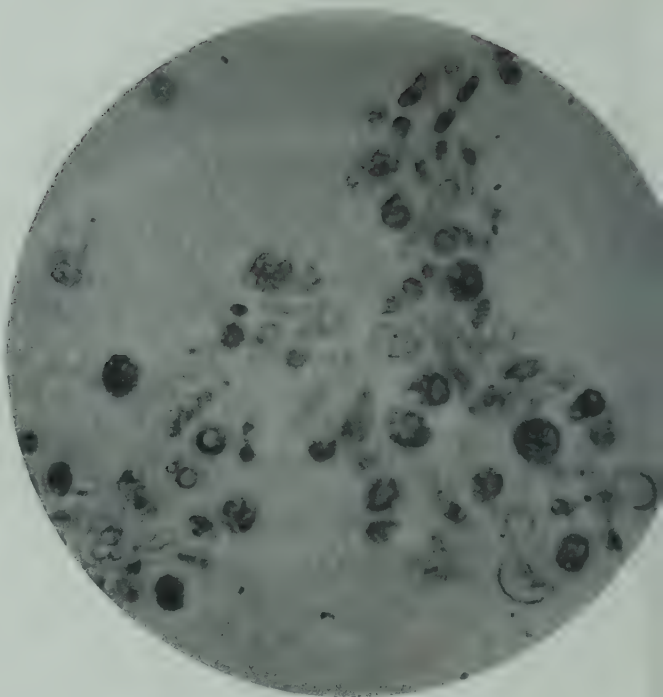
III. l
(8/III)



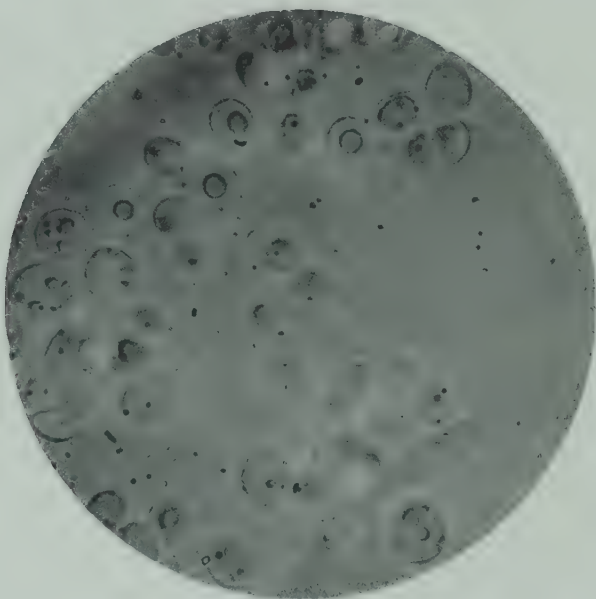
II. c
(8/XI)



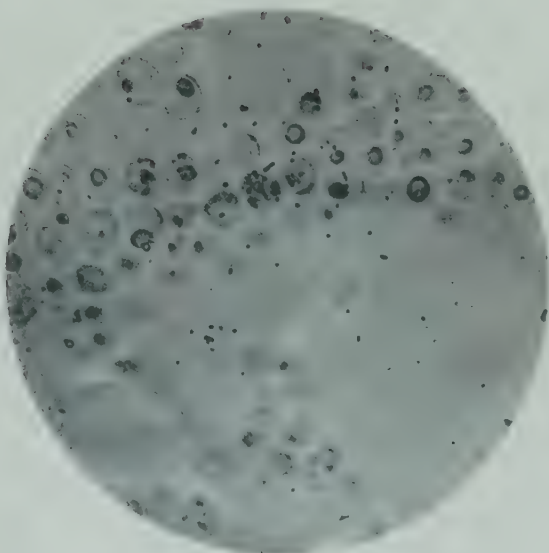
K. s
(15/XI)



II. b
(8/XI)



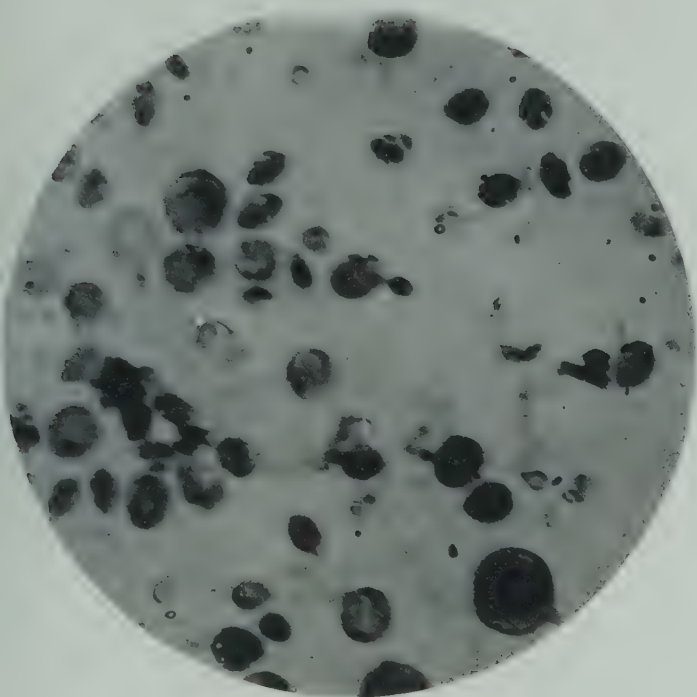
II. a



III. a



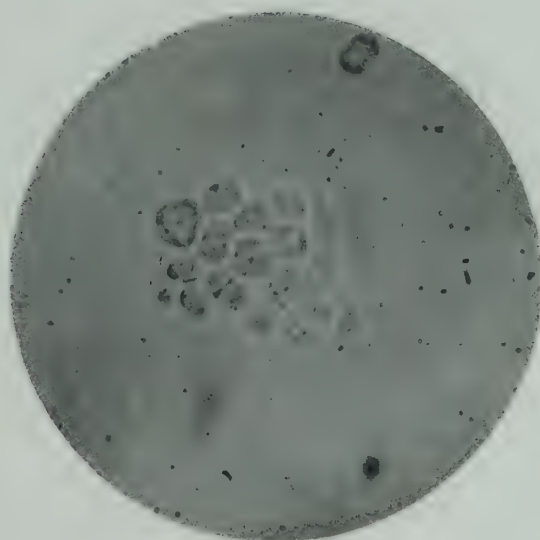
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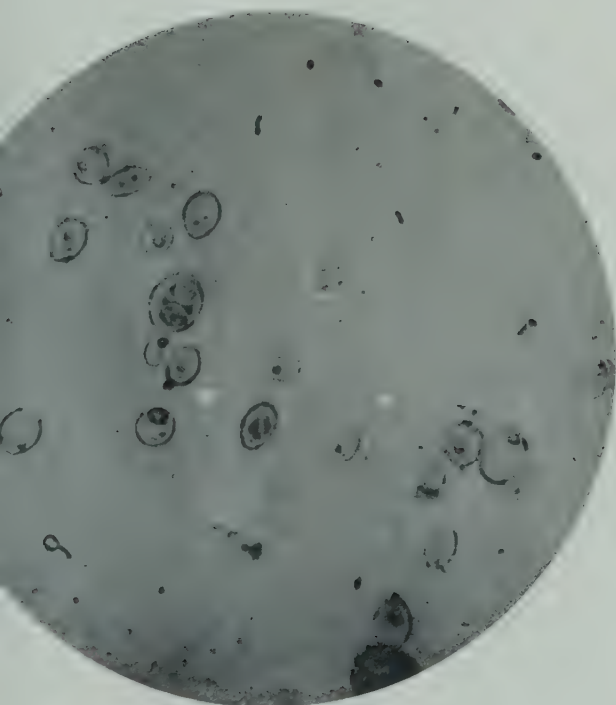
II. *a*
(27/L)



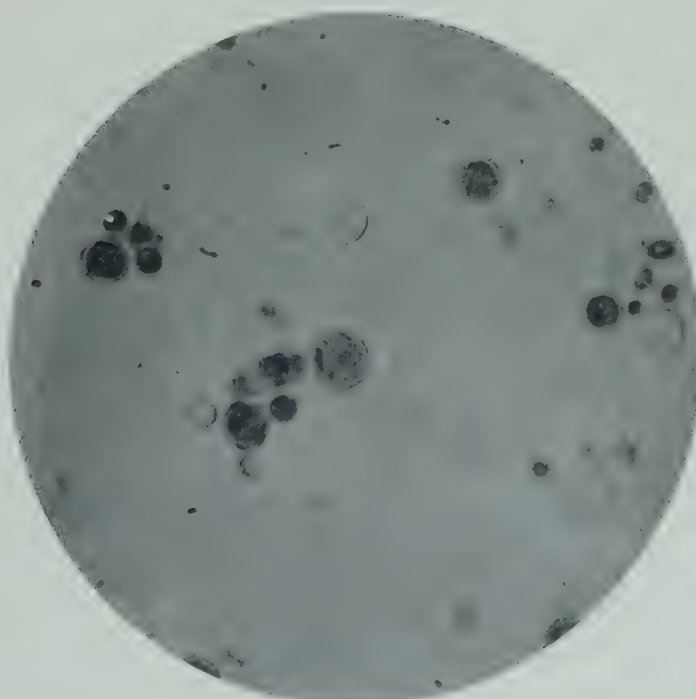
M



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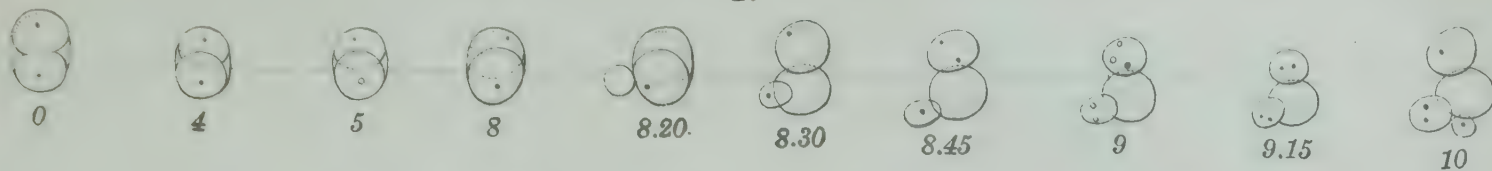


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(1/XI)

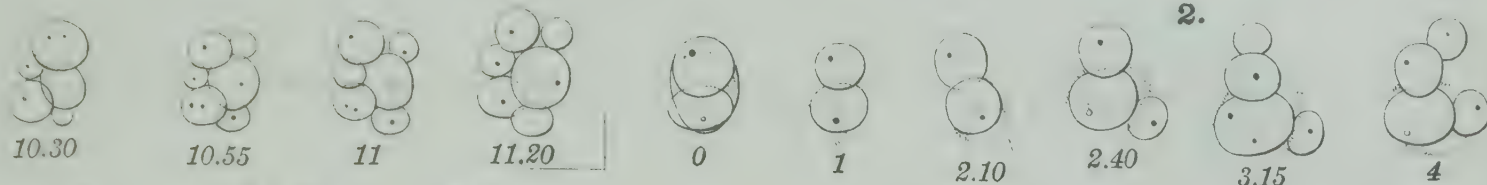


II. *d*

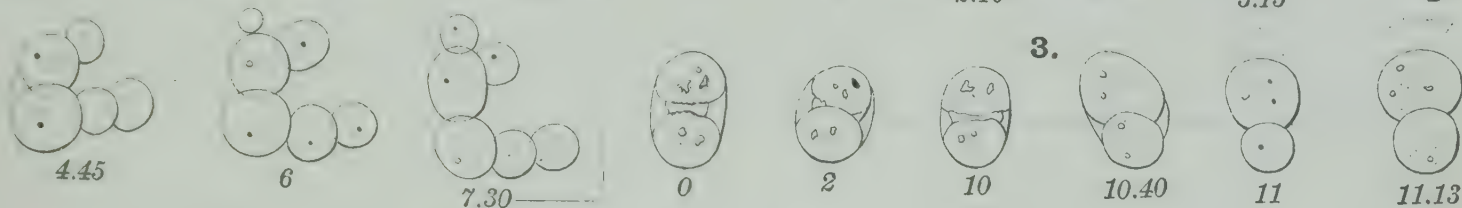
1.



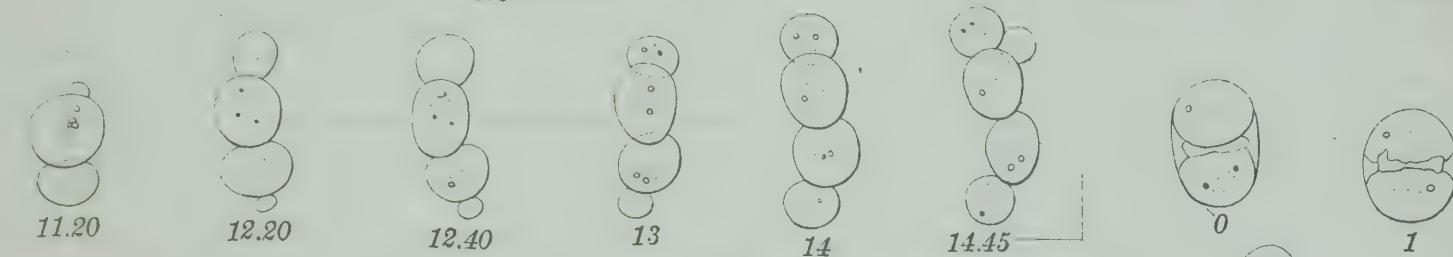
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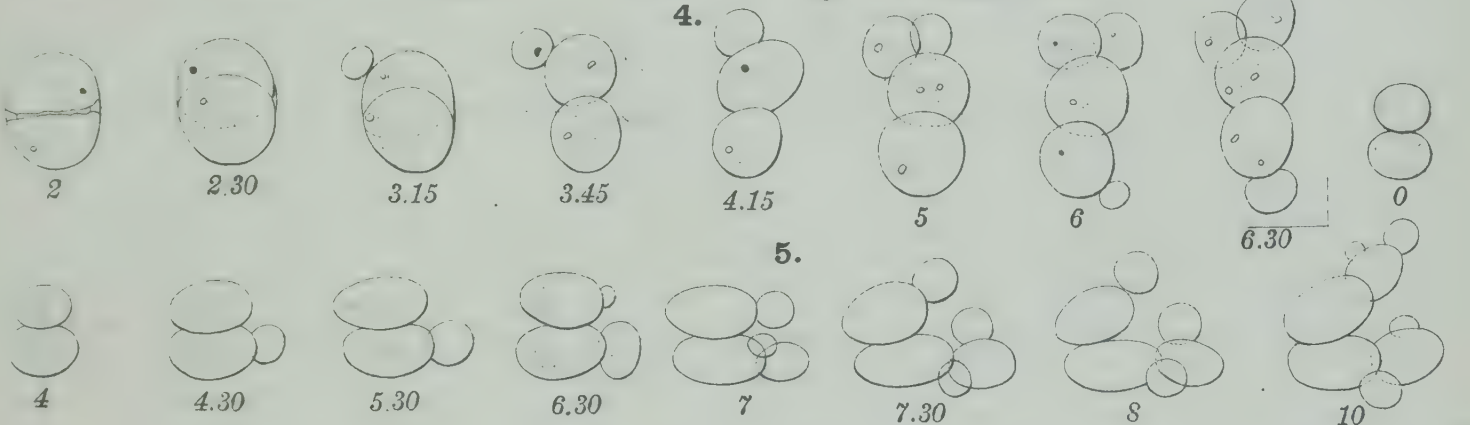
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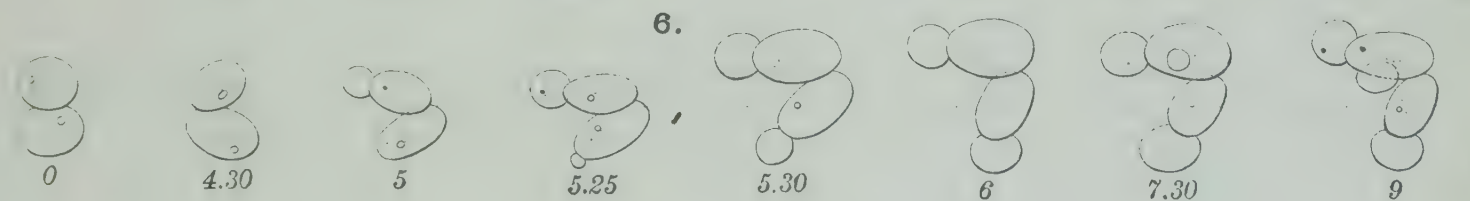
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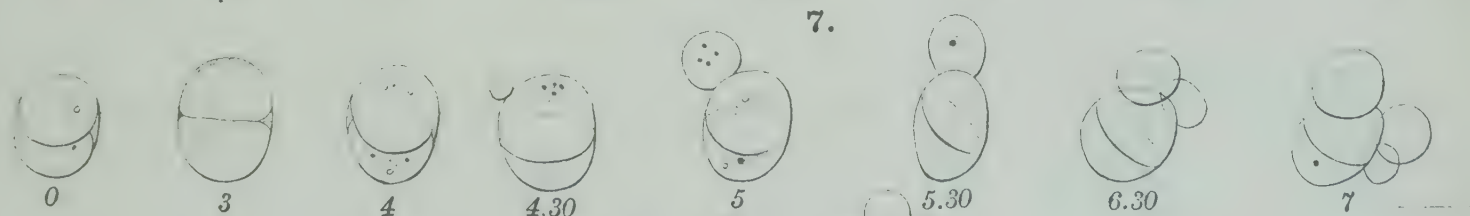
5.



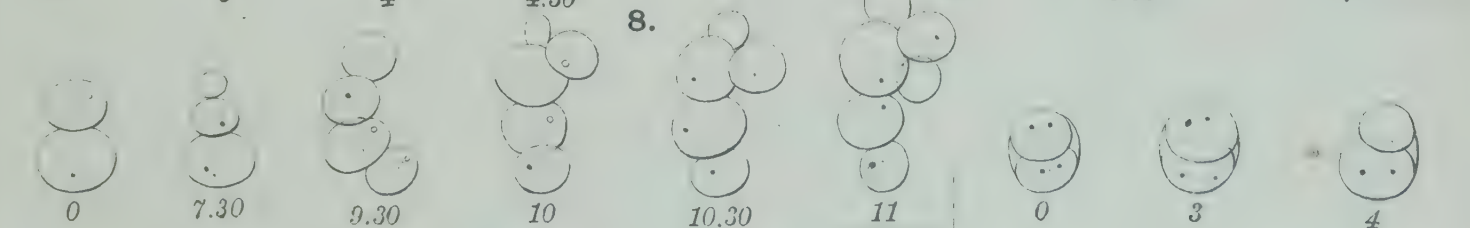
6.



7.



8.



9.





II. 6.

II. a

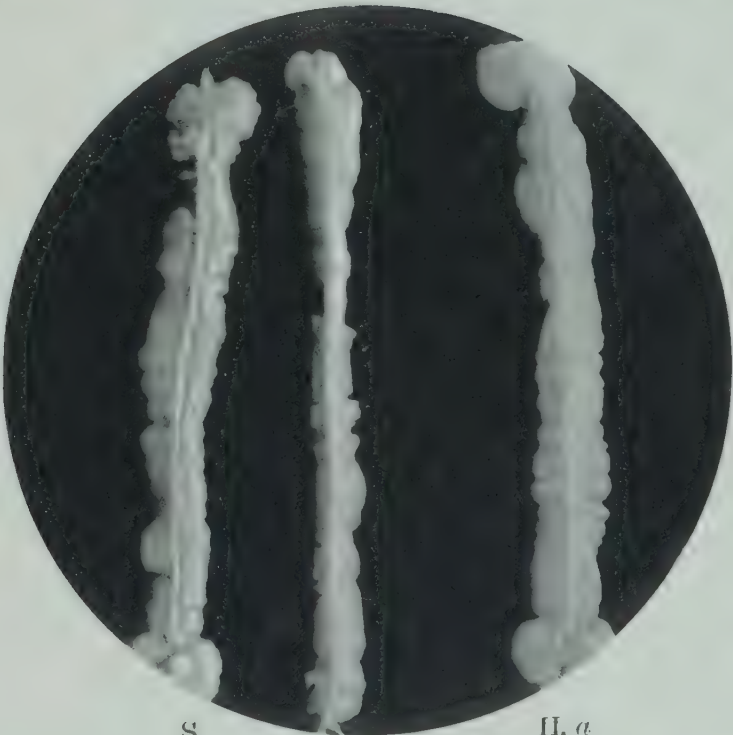
I.



II. c

II. d

III. a



S

I

II. a



III. b

III. c

M



K. s

S

IV. a



IV. a

K. a

n



I

II. a

II. b



II. c

II. d

III. a



III. b

III. c

M

On the Budding Fungi of "Shōyu-Moromi" and "Shōyu-Koji"

BY

Teizo Takahashi and Matao Yukawa.

With Plate XV and 2 Text-Figures.

Introduction.

Up to now several studies on the budding fungi of "shōyu-moromi" and "shōyu-koji" have been published, but with regard to the botanical classification all these studies have very little merit, as we cannot derive any systematical classification from them.

K. SAITO,² in his first report on the microbes of "shōyu," enumerated *Saccharomyces soja*, soy-film-yeast, *Pichia farinosa*, and one species each of *Mycoderma* and *Torula*. Afterwards he gave the soy-film-yeast the name of *Zygosaccharomyces japonicus*³, though the conditions of the cell-fusion and the sporulation of this species have not been fully described. T. NISHIMURA⁴, who published his fruitful investigation on "shōyu," isolated three species of *Torula* under the names of *Torula shoyu*, *Torula shoyu* var. *minuta*, and *Torula turbinata*. According to his investigation these three *Torula* species seem to play an important rôle in the brewing of "shōyu." Further he found several kinds of film forming species, two of which belong to the genus *Pichia* and others to *Mycoderma* and *Torula*. T. MITSUDA⁵ studied five varieties of "shōyu-yeast," but all his yeasts have not the faculty of sporulation.

While G. KITA⁶ found two species of *Torula* and a species which belongs

1. "Shōyu-moromi" (soy mash) is prepared by mixing "shōyu-koji," common salt and water in certain proportion. The preparation of "shōyu-koji" is similar to that of rice or "saké-koji," but in the former steamed soy-bean and roasted wheat are used instead of rice.

2. Cent. f. Bak. II. Ab. XVII. 1906.

3. The Botanical Magazine (Tokyo) XXIII 1909. p. 96. Cent. f. Bak. XXVI 1910. S. 93.

4. Jour. of Scientific Agric. Society (Nōgaku Kwai Hō).

5. Jour. of College of Agric. Tokyo Imp. Univ. Vol. I. No. 3. p. 347.

6. Jour. of Chemic. Indust. (Tokyo) XIV, 156.

[Jour. Coll. Agric., Vol. V. No. 3, 1915.]

to *Saccharomyces*, his description is too short to compare with the preceding studies. From these points of view, we come to the conclusion that "shōyu-yeasts" are not classified systematically. It need not be considered strange that such different analytical results have been stated by several authors, as the samples used for the isolation of yeasts differed from each other, not only as to the factories which supplied the samples, but also as to the kinds and qualities of the raw materials, and further in the manner of preparation and ripening stages of "shōyu-moromi." NISHIMURA, MITSUDA⁷ AND KITA in vain tried spore cultures of their yeasts after the gypsum-block-method, and they regarded their yeasts as *Torula* species. But we believe that there might be a great dissimilarity between the physiological conditions of the budding fungi of "shōyu-moromi", which is distinguished from others by its rich content of common salt, and those of the budding fungi in every other fermenting mash. The necessary favorable conditions, under which even these particular yeasts can as easily produce spores as any other kind of *Saccharomycetaceae*, might be prevented by some unknown factors. Although the specific differentiation of "shōyu-yeast" is not our only object, we believe that it is also important to give a satisfactory solution on this subject for both scientists and manufacturers. Moreover, there are many other points requiring further investigation, so these circumstances led us to make some researches on "shōyu"-yeasts and "shōyu-koji."

PART I. SYSTEMATIC.

Isolation of the Budding Fungi from "Shōyu-Moromi."

Fifty-two samples⁸, at different stages of the ripening process, were taken from eleven different factories⁹ situated in various parts of our country, in order to gather as much as possible all existing kinds of the budding fungi.

To separate the budding fungi from each other in the samples, we started from plate-culture using "shōyu-koji"-agar as the culture medium. Further,

7. MITSUDA did not classify his yeasts from a special point of view.

8. The ages of "moromi" were of different periods, between 2 to 24 months.

9. The following are the factories which very kindly granted us the samples :

SHICHIROYEMON Mōgi's "shōyu"-brewery at Noda in the province of Shimōsa.

the cells developed on the medium from different colonies have been purified by LINDNER's droplet culture.

The following budding fungi were isolated from the samples :

1. *Zygosaccharomyces major*, nov. spec.
2. *Zygosaccharomyces soja*, nov. spec.
3. *Zygosaccharomyces japonicus*, Saito¹⁰.
4. *Zygosaccharomyces salsa*, nov. spec.
5. An asporogenic species of *Zygosaccharomyces* (?).
6. Two species of *Mycoderma*.
7. *Pichia alcoholophila*, Klöcker var.
8. Several species of *Torula*.
9. A species of *Monilia*.

Investigation on the Formation and Germination of Ascospores of *Zygosaccharomyces*.

Forms of cell and spore, production of film, and conditions of reproduction and sporulation, and further enzymatic actions are important factors for the classification of a yeast. Above all, determination of sporulation is perhaps one of the most important factors in the case of the classification of "shōyu-yeast."

Saccharomyces soja, Saito¹¹, seems to be identical with *Torula shoyu*, Nishimura¹² (perhaps also with G. KITA's "shōyu"-yeast No. 1)¹³ in the mycological relations, except that the former ferments galactose and can produce spores, while the latter does not.

SAHEIJI MOGI "shōyu"-brewery at Noda in the province of Shimōsa.

HAMAGUCHI's "shōyu"-brewery at Chōshi in the province of Shimōsa.

G. TANAKA's "shōyu"-brewery at Chōshi in the province of Shimōsa.

J. IWASAKI's "shōyu"-brewery at Chōshi in the province of Shimōsa.

K. NAKAMURA's "shōyu"-brewery at Goyu in the province of Owari.

Asai "shōyu"-brewery company at Tatsuno in the province of Harima.

Maruo-"shōyu"-brewery company at Tatsuno in the province of Harima.

Kikuichi "shōyu"-brewery company at Tatsuno in the province of Harima.

TATSUNO "shōyu"-brewery at Tatsuno in the province of Harima.

Kagawa "shōyu"-brewery experimental station at Shōtoshima in the province of Sanuki.

10. Cent. f. Bak. II. Abt. XVI. 1906.

11. Cent. f. Bak. II. Abt. XVII. 1906.

12. Nōgaku Kwai Hō.

13. Jour. of Chemic. Industry (Tokyo) XIV. 156.

It is necessary to ascertain closely whether galactose is fermentable or not by both yeasts, since the determination of fermentability of galactose is difficult compared with that of any other sugar. To determine the possibility of sporulation of "shōyu-yeast," it is necessary, of course, not only to refer to all the methods recommended for spore culture, but also to modify them, further to establish other suitable methods. Further, a species to which we gave the name of *Zygosaccharomyces soja*, seems to stand in close relation to the preceding two yeasts. Therefore, at first, we started to determine the relation in question from the sporigenical point of view, and we repeated spore culture with this yeast after the following methods: (a) gypsum block-method improved by HANSEN, (b) KLOECKER's gypsum block-method which immerses the gypsum block in wort instead of water¹⁴, (c) SCHOENING's gypsum block-method¹⁵, (d) methods of spreading yeasts on the thin stratum of gelatine, prepared with or without a nutrient solution, likewise in yeast water and in sterilized water, (e) KOHL's spore culture methods¹⁶, (f) BEIJERINK's agar medium method¹⁷, (g) GORODKOWA's method¹⁸. Moreover, we examined the cells not only of old cultures on slices of potato and carrot, but also of the yeast ring or film formed in "koji"-extract, wort and glucose-yeast water-culture. In the preceding six different cultures (a-f) which were kept for 2-20 days at 15°-35°C., a few round, highly refractive, spore-like granules were often observed in abnormal cells; however, these granules were never stained red by double staining. On GORODKOWA's medium which was kept at 28°C., we discovered some dumb-bell-shaped cells after 6 days, and large numbers of small daughter cells which connected together on mother cells after 15 days. Further, we occasionally observed dumb-bell-shaped, ascus-like cells, which contained 1-4 spore-like globules after 3 months. Although we did not try to ascertain, whether these globules are capable of germinating or not, they were easily stained by the double staining method.

Sporulation also has never occurred in the cultures of potato, carrot and

14. *Zygosaccharomyces priorianus*, Kloecker forms large numbers of sporulated cells on this block; Handbuch d. Tech. Mikol., LAFAR, IV. 182.

15. Ibid. 29.

16. Die Hefepilze. Kohl 197.

17. Cent. f. Bak. II Abt. IV. 657.

18. Ibid. XXIV. 318.

glucose-yeast water. Now there is nothing left but "koji 麹" -extract wort-culture to give the last determination for sporulation of this yeast. It will not be useless to give the following details about the cells of the yeast ring developed in "koji" -extract or wort-culture, which were kept for two months at room temperature.

(a) The forms of the cells, from which the yeast ring is constructed, are so various that we could easily observe not only round or dumb-bell-shaped cells, but also highly elongated, mycelial cells, or cells which are similar to the permanent cells and the film cells of the first generation, which WILL¹⁹ had closely investigated with some bottom yeasts.

(b) Sometimes we found a number of round, refractive granules in some dumb-bell-shaped cells.

(c) Most of the cells which formed yeast ring were stained with 0.5% methylen blue solution and GRAM's solution, while the cells which contained a number of refractive spore-like granules were never stained with these solutions.

(d) The dimensions of these granules were so various that the largest one is of the size of 5μ while the smallest one is 0.5μ .

(e) By the double staining method these spore-like granules were never stained intensely red.

Hence we decided to observe, whether these spore-like granules are capable of germinating. Each drop of "koji" -extract, in which a few cells containing the spore-like granules were well distributed, was transferred on a cover glass, and then it was put on a hollowed object glass or BOETTCHER's moist chamber, being sealed with paraffin to prevent the evaporation of water. Such cultures were kept at various temperatures for several days, but they did not change at all.

K. SAITO often observed some sporulated cells in the yeast ring developed in "koji" -extract, in which he cultivated his "shōyu" -yeast, but he says nothing about any other conditions which have influence on the sporulation. Therefore, to determine more closely the individual conditions which influence

19. Handb. f. Tech. Mykol. LAFAR. IV. 17-18.

the sporulation of our yeast, we cultured the yeast in "koji"-extract for 3 or 6 months at various temperatures extending from 17° to 25°C.

The cells which are similar to the sporulated cells of SAITO's yeast have very rarely been observed in some yeast rings, and spore-like globules which occur in these cells were distinguished from the round, refractive granules as already mentioned. These cells showed also the forms of dumb-bell or like bodies. The globules which occurred in a mother cell were always transparent, round or oval, and contained a few tiny grains respectively.

In such respects these cells seem to coincide with the sporulated cells of SAITO's yeast, but unfortunately, since these ascus-like cells were discovered too rarely to observe their germination, we were obliged to satisfy ourselves only by trying the double staining method for spore-determination. According to this double staining only the content of spore-like globules was stained red, and it was clearly distinguished from its wall.

Hence it is justifiable to conclude that specific differentiations of "shōyu"-yeasts have not coincided with one another, owing to their sporulating difficulties.

Now after trying all these methods, we must discover some favorable conditions, under which even this obstinate yeast can easily produce its spores.

When we observed the cells of a yeast ring developed in diluted "shōyu" culture²⁰ of *Zygosaccharomyces soja*, we happened to recognize large numbers of dumb-bell-shaped cells, which contain 1-4 transparent spore-like globules. Moreover, it was clearly brought to light that these particular globules are real ascospores, by culturing these dumb-bell-shaped cells in BOETTCHER's moist chamber, which was laid under a microscope in the warming apparatus (at 35°C). In these germinal cultures we observed that these globules swell up to a considerable size, put forth a bud and henceforward behave like vegetable cells (see Text-figure 2 on page 226). Moreover, these globules were always stained red by the double staining method, and yellowish blue with GRAM's solution.

20. "Shōyu" (which commonly contains 15-17% NaCl) was diluted with water to make its salt content 5%, and was sterilised intermittently in Koch's steriliser for 3 days. After the inoculation of *Zygosaccharomyces soja* it was kept in a thermostat at 22°C. for 15 days.

It is a remarkable fact that every spore of all the *Zygosaccharomyces* isolated from "shōyu-moromi" stains yellowish blue with GRAM's solution.

In order to decide whether the sporulation of "shōyu"-yeast is influenced by dilutions of "shōyu," contents of salt, or nutrient fluids, and further by temperatures, we kept the cultures at various temperatures, using "koji"-extract or diluted "shōyu," which contained different quantities of salt. These experiments showed that sporulation of this yeast occurs earliest in yeast ring developed in sterilised diluted "shōyu," which was made to contain 4-5% salt by adding a sufficient quantity of water.

But it must be borne in mind that the sporulation of this yeast is influenced by the dilution of "shōyu" and the temperature of the culture.

We observed large numbers of sporulated cells in the diluted "shoyu"-culture, which was kept at 28° C. for the first 3 days and then at 20°-25° C. for 10 days. On the contrary, sporulation has not been observed in the same medium which was kept always at 28° C. for 20 days. In the case of "koji"-extract which contains 2-7% NaCl, being treated under the above conditions, sporulation has also been observed in the yeast ring after the same number of days, but the number of sporulated cells was less than that in the case of diluted "shōyu."

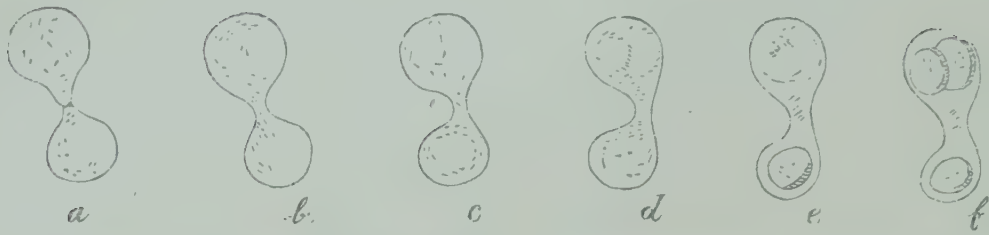
Therefore, if it be desired to study the process of sporulation of "shōyu"-yeast, it should be undertaken in the following ways.

(1) The yeast is sowed in a sterilised test tube which contains a quantity of diluted "shōyu" (5% NaCl), and the culture is laid at 28° C. for the first 3 days and then steadily kept at 20°-25° C. for several days.

(2) When well defined yeast ring develops along the wall of the tube, a few parts of the ring are examined under a microscope. As soon as a number of beak-shaped cells is observed, a small portion of the ring must be distributed in water in a sterilised PETRI-dish.

(3) A cover glass, on which the water is dropped, is put in BOETTCHER's moist chamber, and sealed with paraffin to prevent the evaporation of water.

(4) It is observed under a microscope keeping it at 20° C. According to the above treatments we easily find that sporulation of our *Zygosaccharomyces* occurs in the following manner:



Text-fig. 1.—*Zygosaccharomyces japonicus* Saito. Ascosporeulation. For explanation see text.

Two cells close to each other force up beak-like tubes and fuse together by growing up their length against each other, and accumulate their plasmic granules towards the contact canals (glycogen reaction is especially remarkable in this contact canal). This is shown in Text-fig. 1, *a*. The wall in the contact canal breaks through of itself, and the plasma in one side or both sides of the ascus separates into a number of vacuole-like balls, which correspond to spores, as shown at *b*—*e*. Since these balls become surrounded with the plasmic granules, the process of sporulation is not distinctly visible. The spores are matured after 24 hours; they contain a few tiny grains and are transparent, round or oval (see *f*).

Although the total number of the spores in each ascus is 1–4 (mostly 4), the number of spores which occurs in each part of an ascus are very variable.

It is not rare to find that spores occur only in one part of an ascus. In this case, sometimes a few portions of the plasma of other parts remain in the contact canal, as if the remaining plasma were drawn into the one part which produced the spores.

A. NON FILM-FORMING ZYGOSACCHAROMYCES.

I. *Zygosaccharomyces major*, nov. spec.

Since this yeast was mostly isolated from the samples of mature stage, it seems to play an important rôle in the ripening of “shōyu.”

1. FORM AND SIZE. In “koji”-extract or wort culture (4 days at 20° C.) cells are mainly spherical (3.7–7.5 μ), sometimes oval, and their contents homogeneous and sometimes exhibit vacuoles. The glycogen reaction is evident in every cell. Cells in yeast ring of “koji”-extract culture (after 20 days at 20° C.) are so irregular that a small cell may be 2.5 μ while larger ones mea-

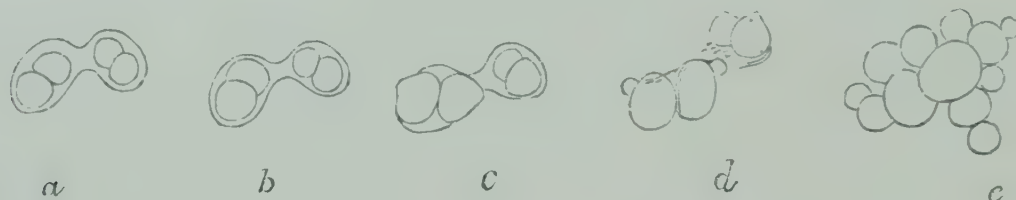
sure up to $10\ \mu$. The occurrence of these cells seems to be somewhat prolonged in wort or "koji"-extract which contain a quantity of salt. Old culture in the same media (2-6 months at room temperature) exhibits not only the cells which are similar to Will's film cells of the first generation, and permanent cells, but also very highly elongated, mycelial ones.

2. GROWTH. (a) Solid culture: Plate culture (7 days at room temperature): In "koji"-extract or wort gelatine this species forms white greyish, round, bright waxy colonies. Streak culture: On "koji"-extract-agar (30 days at 27°C .) it grows with somewhat brownish, waxy, dull lustered, elevated covering. Margin shows somewhat paralleled streamy canals. On glucose-saké-agar (10 days at 25°C .) it forms a greyish white, waxy covering with slowly elevated sides. The central part is somewhat concave and the marginal part dull toothed. Stab culture: On "koji"-extract gelatine (30 days at 15°C .) it forms waxy, feeble lustered, brownish, elevated isles at the mouth of the stab canal, and rosary-like colonies with gas bubbles along the canal. (b) Fluid culture: This species grows in many fluid media. According to the appearance of its fermentation it belongs to the so-called bottom yeasts. In "koji"-extract culture (at 25°C .) yeast ring appears first after 3 days, but it does not form any complete ring even after 6 months, while the sedimental yeast crop becomes somewhat plenty after 3 weeks. Its development in wort or hopped wort seems to be inferior to that in "koji"-extract. Its resisting power against NaCl is so striking that it can grow tolerably in "koji"-extract or wort containing 20% NaCl.

3. BEHAVIOUR TOWARDS SUGARS. This was determined with LINDNER's method. This species ferments dextrose, laevulose, mannose, saccharose, maltose, but not galactose, lactose, raffinose, α -methyl-glucosid.

4. FORMATION and GERMINATION of SPORE. This species is one of the easily sporulable kinds among all the *Zygosaccharomyces* isolated from "shōyu-moromi." This yeast does not form spores on gypsum-block at all. Sporulated cells occur very rarely in yeast ring developed in "koji"-extract culture (3-6 months at 25°C .) or on GORODKOWA's agar medium (20 days at 25°C .) On the other hand, following the diluted "shōyu"-culture which has been described in the preceding page, we see large numbers of ascus easily occur in the yeast ring within 7-15 days. The processes of formation and germination

of spores are similar to those of *Zygosaccharomyces soja* which have been already described (see pages 234 and 236). Spores are transparent, round or oval, commonly 3-4.5 μ . A few tiny grains are contained in each spore. The total number of spores in each ascus is 1-4, but the number of spores which occurs in each part varies.



Text-fig 2.—*Zygosaccharomyces major*, nov. spec. Germination of spores. *a*, a parent-cell with four spores; *b*, the same after seven hours in koji-extract at 30°C in Böttcher's chamber; *c*, after ten hours, each two spores carry a single bud; *d*, after twelve hours, the membrane of the parent-cell, having been torn, and the other two spores each putting forth a bud; *e*, after twenty-two hours.

5. AFFINITY. This species seems to be nearly similar to *Torula shoyu* var. *minuta*, which was isolated from "shōyu-moromi" by J. NISHIMURA. It is necessary to ascertain the sporulation of the latter yeast after our method.

This yeast differs distinctly from *Zygosaccharomyces soja* and the asporogenic species of *Zygosaccharomyces* by the following characteristics:

This species ferments saccharose, and the time required for sporulation is far shorter; the number of sporogenic cells in the yeast ring is always abundant.

Zygosaccharomyces salsus distinguishes itself from this yeast by the formation of a particular film.

As far as we know, there are seven species of *Zygosaccharomyces*, as follows:

- (a) *Zygosaccharomyces Barkeri*, Saccardo and Sydow.²¹
- (b) *Zygosaccharomyces priorianus*, Kloecker.²²
- (c) *Zygosaccharomyces javanicus*, Krnyff.²³

²¹Handbuch d. Tech. Mykol. LAFAR. VI. 182.

²²Ibid.

²³Centralbl. f. Bak. II Abt. XXI. 619.

- (d) *Zygosaccharomyces lactis* α , Dombrowski.²⁴
- (e) *Zygosaccharomyces japonicus*, Saito.²⁵
- (f) *Zygosaccharomyces fusoriens*, Saito.²⁶
- (g) *Zygosaccharomyces* from cacao.²⁷

Only the characteristic points in which these species differ from *Zygosaccharomyces major* will be given in the following lines :

(a) The cells of *Zygosaccharomyces Barkeri* are oval and large, and sporulate on gypsum block, on several solid media containing wort or "koji" -extract and on damp-bread, potatoes, etc., further, it does not ferment maltose.

(b) *Zygosaccharomyces priorianus* forms cells of various shapes in young wort culture, and large numbers of ascus on the surface of wort-gelatine, on sterilised carrot slices, and on gypsum block, that have been immersed in wort instead of water. Moreover, this species does not ferment saccharose.

(c) The cells of *Zygosaccharomyces javanicus* are oval. This species ferments galactose, and forms large numbers of sporulated cells on agar at 26° C.

(d) *Zygosaccharomyces lactis* α forms spores easily, further it ferments lactose but not maltose.

(e) *Zygosaccharomyces japonicus* produces immediately a greyish film on the surface of some nutrient fluids, and does not ferment saccharose.

(f) *Zygosaccharomyces fusoriens* does not ferment saccharose.

(g) *Zygosaccharomyces* from cacao does not ferment saccharose.

According to the above distinctions this yeast is doubtlessly a distinct new species, and we give it the name of *Zygosaccharomyces major*.

II. *Zygosaccharomyces soja*, nov. spec. (*Saccharomyces soja*, Saito [?]).

This yeast was mostly isolated from "shōyu-moromi" which was at a young stage of the ripening process, and seems to be an important species for "shōyu"-manufacture. Excepting the fermentability of galactose, *Sac-*

²⁴Centralbl. f. Bak. II Abt. XXVIII, 371.

²⁵Centralbl. f. Bak. II Abt. XVII, XXVI.

²⁶Woch. f. Bran. 1911 Nr. 6. LINDNER.

²⁷Ibid.

Saccharomyces soja seems to be similar to this yeast. Moreover, there is not a great difference between *Torula shōyu* and this yeast. According to SAITO's illustration it is questionable that he, who gave the name of *Zygosaccharomyces japonicus* to his "shōyu"-film-yeast, included his "shōyu"-yeast in the genus of *Saccharomyces*.

JOERGENSEN²⁸ makes the same inference on this question.

1. FORM AND SIZE. Young cells from "koji"-extract or wort-culture (5 days at 20° C.) are commonly spherical or oval, 3.5-8 μ in diameter. The contents are homogeneous and exhibit sometimes vacuoles, and are rich in glycogen. The cells of old cultures (after 2-6 months) in "koji"-extract or wort are already described and are almost the same as in *Zygosaccharomyces major*.

2. GROWTH. (a) Solid culture: On "koji"-extract-gelatine-plate this yeast forms bright pearly, greyish white, mostly round and elevated colonies. Streak culture: (1) "koji"-extract agar (at 27° C.): This species forms a greyish white, waxy, elevated surface, but after a month it becomes somewhat brownish and the centre of the growth flat. The marginal part shows tooth-like engravings. (2) Glucose-"sake"-agar: The growth shows yellowish white, waxy lustre, and forms an elevated smooth surface with fine streaming along the track. The marginal part is somewhat uneven. Stab-culture: The growth is the same as with the preceding species, but the surface of the isle is more concentric. (b) Fluid culture: The appearance of development of this species is very similar to that of *Zygosaccharomyces major*. This species can also reproduce and ferment in every nutrient fluid which contains 20% NaCl.

3. BEHAVIOUR TOWARDS SUGARS. This yeast ferments dextrose, laevulose, maltose, mannose, but not saccharose, raffinose, galactose, lactose, α -methyl-glucosid.

4. FORMATION AND GERMINATION OF SPORE. On these relations we have already written fully. Form and size of spores of this species are similar to those of *Zygosaccharomyces major*, but the numbers of sporogenic cells are always less than in the latter species. Moreover, the time required for

²⁸Die Microorganismen d. Gärungsindustrie. IV. Aufl. JOERGENSEN 370.

the occurrence of sporulation is longer than that of *Zygosaccharomyces major*.

5. AFFINITY. This species does not ferment Saccharose, but *Zygosaccharomyces major* attacks the same sugar well, and both species are easily distinguished from each other by the dimensions of the cells and the growths on glucose-"saké"-agar.

This species differs from *Zygosaccharomyces Barkeri* by the sporogeneticality and in its behaviour towards maltose, and from *Zygosaccharomyces priorianus* by the cell forms of young culture and the circumstance of sporulation. *Zygosaccharomyces javanicus* is easily distinguished from our yeast by the size of cell, the fermentability of galactose and the formation of large numbers of spores on agar. *Zygosaccharomyces lactis* α ferments lactose but not maltose. *Zygosaccharomyces japonicus* produces easily a particular film on the surface of nutrient fluid. Neither *Zygosaccharomyces fusoriens* nor *Zygosaccharomyces*²⁹ from cacao ferment saccharose like our yeast, but both species ferment dextrine strongly.

On the other hand, *Saccharomyces soja* and *Torula shoyu* seem to stand in close relation with our yeast, however, it might be appropriate to comprise these three yeasts into one and the same species. Be that as it may, we will give it the name of *Zygosaccharomyces soja*.

III. Asporogenic species of *Zygosaccharomyces* (?).

We have hardly ever met with this yeast in our investigations. On the mycological relations it is nearly similar to *Zygosaccharomyces soja*. This yeast forms well defined, yeast ring in "shōyu" and "koji"-extract, but the sporulated cells have never been observed in any yeast ring in spite of the presence of a number of dumb-bell-shaped cells.

According to this, this yeast seems to be a variety of *Zygosaccharomyces soja*, which has lost the capacity of forming spores. Subsequently we have continued to cultivate this yeast in various nutrient media for restoring the power of sporogenation. Whether this yeast has lost the faculty of producing spores temporarily or entirely will be reported hereafter.

²⁹There is nothing reported about the behaviour of these two yeasts towards sugars, so we will disregard them in this case too.

B. FILM FORMING ZYGOSACCHAROMYCES.

I. *Zygosaccharomyces japonicus*, Saito.

This species was isolated from many samples, especially from all the samples of Hamaguchi's factory. Since this yeast and *Zygosaccharomyces salsa* develop and form particular greyish brown films even on a concentrated "shōyu," which any other kind of film forming yeast could no more grow, both these yeasts are most feared in the storing of "shōyu." Moreover, this species forms very easily a large number of sporulated cells.

1. FORM AND SIZE. Young cells from the surface cultures on "koji"-extract agar are round (commonly 4–8 μ) or oval, and contain glycogen. In old cultures club-shaped or mycelial cells are often observed. Most of the cells in a diluted "shōyu" are abnormally elongated with an increased number of vacuoles.

2. GROWTH. Solid culture: On plate-culture of wort-gelatine it forms greyish white, crater-like, elevated colonies with smooth periphery, and the colour turns brownish after lapse of time. Streak culture: On "koji"-extract-gelatine: The growth shows a greyish white, somewhat dried, lustered, folded covering with fine toothed margin. Fluid culture: "Koji"-extract culture (at 23° C.): It forms mealy white, small filmy fragments on the surface, and covers the whole surface after 3 days. The film crinkles like crêpe paper, increasing its thickness, and its colour changes into yellowish brown. After 3 weeks the film sinks gradually and deposits a great deal of sediment on the bottom, leaving a thin film over the surface, and at last only a few parts of the yeast ring remain along the wall. Wort culture (at 23° C.): In the culture which has been kept for 7 days the film is not yet formed, although gas bubbles ascend through the medium. After 3 months well defined yeast ring and thin film become observable, but this film never folds at all. This species reproduces and forms its particular film even in "koji"-extract or "shōyu" which contains 23% of NaCl.

It is most noticeable that this species forms a greyish-brown, folded film on the surface of sterilised "shōyu" after a long time, while other races

which we isolated from "shōyu-moromi" cease the reproduction of their cells in the same medium.

3. BEHAVIOUR TOWARDS SUGARS. This species ferments dextrose, maltose, laevulose, but not saccharose, lactose, raffinose, α -methyl-glucosid, galactose.

4. FORMATION AND GERMINATION OF SPORE. This species rarely produces spores in yeast ring of "koji"-extract-culture (after 3 months). After GORODKOWA's method sporulation occurs often after 10 days at 28° C. Following the diluted "shōyu" method which has been described in the preceding page, a large number of sporulated cells occur in the yeast ring after 4-5 days. The spore has a somewhat thick wall and contains a few tiny grains; it is transparent, round or oval and 2.5-6 μ in size. The processes of formation and germination of the spores of this yeast, and other relations are also similar to the preceding yeasts.

5. AFFINITY. This yeast seems to be identical with *Zygosaccharomyces japonicus*, Saito.

II. *Zygosaccharomyces salsus*, nov. spec.

This species was discovered in the samples taken from all the factories at Tatsuno.

1. FORM AND SIZE. Young cells from the surface culture on "koji"-extract agar are mostly round (4-8 μ) or rarely oval. The contents are homogeneous and exhibit sometimes vacuoles.

2. GROWTH. Solid culture: Streak culture (27° C.): The growth shows a greyish white, feeble, finely folded covering with somewhat steep sides. Glucose-"sake"-agar (10 days at 25° C.): It forms a greyish yellow, folded, steeply elevated covering with streamy margin. Fluid culture: "Koji"-extract culture (at 23° C.): It forms a few parts of yeast ring without clouding the fluid after 3 days. The ring grows gradually and increases in thickness. After 3 weeks a thin film covers the surface. The culture medium which was kept for 3 months has been strikingly decolorised.

Wort culture is similar to the former culture. But this yeast forms a greyish white, folded, thick film on "shōyu" or "koji"-extract which con-

tains a quantity of NaCl. This yeast is easily distinguished from *Zygosaccharomyces japonicus* by this characteristic point.

3. BEHAVIOUR TOWARDS SUGARS. This species ferments dextrose, laevulose, maltose, but not galactose, lactose, saccharose, raffinose, α -methylglucosid.

4. FORMATION AND GERMINATION OF SPORE. In these relations this yeast is similar to *Zygosaccharomyces japonicus*, but the time required for sporulation of this yeast is longer than that of the former species.

5. AFFINITY. This yeast forms a folded thick film in some nutrient fluids which contain a quantity of NaCl, but not in the absence of NaCl. Moreover, this yeast is easily distinguished from the former species by the cell forms,³⁰ and the time limit of sporulation.

NISHIMURA's *Torula soja*, *T. G.* and *T. H.*³¹ seem to be identical with this species. From the above differentiation we gave it the name of *Zygosaccharomyces salsa*.

C. MONILIA.

This fungus was isolated from a young "moromi", which was only three months old. Although a species of the genus *Monilia* was isolated from HAMAGUCHI's "shōyu-koji" by K. SAITO, its morphological description has not yet been written. According to T. NISHIMURA's investigation a species of *Monilia*, which was named *Monilia koji* by him, seems to be distributed in the majority of "shōyu-koji", which were collected from numerous "shōyu" factories situated in various parts of our country. But his description also lacks sufficient mycological observations to recognise it as a new species.

1. FORM AND SIZE. This fungus has an extensive POLYMORPHISM. In aged culture it always shows very elongated hyphae and an intensive mixture of torulaceous conidia and oidium cells. When grown on "koji"-gelatine or "koji"-agar and in yeast water it appears mainly as cells of various size and shape, from globular to ellipsoidal. In wort or "koji"-extract culture it exhibits chiefly these forms in the sediment, but the film cells consist principal-

³⁰Film cells of *Zygosaccharomyces japonicus* are always more or less prolonged.

³¹Nōgaku Kwai hō.

ly of elongated filamentous buds together with oidia and yeast conidia, whereas in hopped wort culture elongated cells predominate.

The protoplasmic content of the cell is pale and homogeneous. One or three fat globules are always seen in the aged cells of all the cultures, which are large vacuoles present in the cell, containing one spheroidal granule which is in constant rapid motion. The glycogen is evident in every cell.

2. GROWTH. (a) Solid culture: On "koji"-extract-agar plate it forms greyish white waxy and somewhat elevated colonies with corrugated surface. Streak culture on "koji"-extract-agar shows a yellowish white covering which is composed of mesentery-like folds, but in time the covering alters to a brownish yellow sponge-like mass. On "koji"-gelatine it forms a similar covering, but the outer portions exhibit fibrous forms.

(b) Fluid culture: In "koji"-extract (at 25-28°C) it forms at first a yellowish white, loose, folded film. After two weeks the film increases in thickness and deposits a great deal of sediment on the bottom, so that the whole of the fluid is finally almost completely filled with masses of the fungus. The filamentous growth is easily seen on the film which ascends the wall. The development in wort or hopped wort is somewhat less than that in "koji"-extract. It grows very slightly in yeast water with a thin film and some sediment. The fungus can even *develop tolerably in every saccharine fluid which contains 22% NaCl*. The inoculated diluted "shōyu" which was kept at room temperature for eight months, has been *strikingly decolorised* and has shown alkaline reaction.

3. BEHAVIOUR TOWARDS SUGARS. It ferments dextrose, laevulose, mannose, galactose, saccharose and raffinose, but no maltose, lactose, rhamnose, inulin, α - and β -methylglucosid.

In "koji"-extract the production of alcohol is very small owing to the formation of esters.

4. FORMATION OF SPORE. Sporulation has neither occurred on gypsum block nor in diluted "shoyu" at any temperature. Both the old yeast ring and the aged film were also vainly tried for sporulated cells.

5. AFFINITY. According to the diagnostic descriptions this fungus seems to be a species of the genus *Monilia*. We have numerous reports of the

fermenting species of *Monilia*, but we can only number *Mon. candida*, *Mon. variabilis*, *Mon. javanica* and *Mon. sitophyla* as clearly identified species, the latter, however, differs distinctly from our species in the morphological side. Consequently we have tried the following investigations with our species and the other three cultures which are kept in our laboratory.

(a) Cultures in cowdung-extract (at 25°C). Our species forms a similar thin disintegrable film to those of *Mon. candida* and *Mon. javanica*, whilst *Mon. variabilis* grows only in the bottom of the fluid. The film cells of both our fungus and *Mon. candida* exhibit delicate filamentous mycelia and branched hyphae, but the mycelial cells of the latter are always thicker and longer than those of the former. However, *Mon. javanica* appears always as globular cells in the same fluid.

(b) Cultures in "koji"-extract (at 25°C). Whilst our fungus takes after *Mon. candida* closely in its film, *Mon. variabilis* produces a thick film which puts forth a number of tufted growths extending downward in the fluid, and *Mon. javanica* forms a white thin crinkled film on the surface and produces a vigorous disagreeable ester-like flavour.

(c) Fermentation faculty. In regard to the fermenting properties our fungus may be distinguished from *Mon. candida* by not fermenting maltose and α -methylglucoside, and from *Mon. variabilis* by the behaviour towards β -methylglucoside and mannose since it is diametrically opposed to that of our fungus, further from *Mon. javanica* by not fermenting maltose, α - and β -methylglucoside.

(d) Action on saccharose. Some 10% saccharose solutions were put in two test tubes, and repeatedly sterilised. The one of them was inoculated with our fungus and the other with *Monilia Candida*, and both solutions were kept at 30°C for 2 days. Then each solution was filtered and separated into two portions. The one portion was boiled with FEHLING's solution, and the alcohol test was made with the other portion. The solution which was inoculated with our fungus gave iodoform reaction and reduced FEHLING's solution, however, the other solution gave iodoform reaction only without the reduction of FEHLING's solution. This investigation shows that unlike *Monilia candida*, our fungus is capable of inverting saccharose in the outside of the cell. So our fungus may be easily distinguished from *Monilia candida* in this respect.

(e) Cultures in NaCl containing "koji"-extract (at 25°C). Whilst our fungus grows tolerably and forms a crinkled film on the surface of "koji"-extract which contains 18% of NaCl after three days, *Mon. candida* develops slowly and commences to form filmy isles on the surface. Both *Mon. javanica* and *Mon. variabilis* are hardly able to form any film even after two weeks.

(f) All species can develop on "koji"-gelatine at 4°C; however, *Mon. candida* and our fungus grow more slowly than the other two. On the other hand, *Mon. candida* thrives even at 42–43°C, but the *maximum temperature for the vegetative process of our fungus is 39–40°C*. Putting these investigations together, it can be inferred that this *Monilia* represents a new species. And our species seems also to be distinguishable from a new genus, *Pseudomonilia*, by some morphological and physiological points.

D. PICHIA.

Pichia alcoholophila, Klöcker var.

This yeast isolated from one sample of "shōyu-moromi" is also one of the species detrimental to the storing of "shōyu," because it easily forms dirty white, folded films on the surface of shōyu.

1. FORM AND SIZE. The cells of this species are mostly sausage shaped, elongated, seldom oval or spindle shaped, measuring 3.5–20 μ in length by 1.5–3 μ in breadth.

2. GROWTH. Streak culture on "koji"-agar shows dried, lustreless and folded coating with somewhat elevated margin. On "koji"-extract it forms rather moist, thin folded films which display white, dried spots.

3. BEHAVIOUR TOWARDS SUGARS. This species ferments glucose very doubtfully, but not fructose, maltose, saccharose and lactose.

4. FORMATION OF SPORE. The spores are spherical, 2–5 in a cell and 1.5–2 μ in diameter. They are produced both on gypsum-blocks (within 2 days at 25°C.) and in the films.

5. This species does not liquify gelatin.

This yeast seems to be a variety of *Pichia alcoholophila*, Klöcker²².

E. TORULA α . (4. 31. cf. Part II).

This fungus was isolated from "shōyu-moromi" of both mature and young stages of the ripening process. Since this fungus develops well in "shōyu" and forms a thin film on the surface, and further decolorises it, it is also detrimental to the storing of "shōyu."

1. FORMS AND SIZE. This fungus develops globular or oval cells, but spindle shaped cells, tapering off at both ends, are not uncommon. Ring cells grown in "koji"-extract or wort exhibit also globular, oval or sausage shapes. One or two highly refractive granules appear in every old cell. Beyond these cells very remarkable gigantic cells, the dimensions of which greatly exceed the average size, are always found in very aged ring. This characteristic cell exhibits a round fan shape of which the short handle is slightly twisted.

2. GROWTH. (a) Solid culture: On "koji"-extract agar plate (at 25°C for 5 days) it forms greyish white, somewhat elevated and waxy colonies with smooth periphery. Streak culture on the same medium (at 20°C for 4 days). The growth along the track is somewhat elevated, moist lustrous, yellowish brown and the margin is wavy. Gelatine is tolerably liquified by this fungus. (b) Fluid culture. This fungus covers "koji"-extract with a thin dull greyish film at the end of twenty four hours. Then the fluid becomes cloudy with the formation of ropy sediments and evolution of carbonic acid. The ring gradually increases its thickness and becomes lemon-yellow. Its development in both wort and hopped wort are inferior to that in the preceding medium. However, this fungus imparts the strongest lemon-yellow coloring to hopped wort. "Shōyu" seems to be a favourable food stuff for this fungus. After eight months the inoculated "shōyu" is *decolorised to a greater extent* and shows *marked alkaline reaction*. This fungus is capable of developing in "koji"-extract which contains twenty per cent NaCl.

3. BEHAVIOUR TOWARDS SUGARS. This fungus splits up glucose, laevulose, maltose and saccharose but not galactose, lactose, α -methylglucosid.

4. FORMATION OF SPORE. Sporulated cells have never been seen in any spore culture in spite of many trials, hence this fungus seems to reproduce by budding only.

Summing up we see that this fungus belongs to the Torulaceae.

F. TORULA β . (21, 12, 37. ff. Part II).

This fungus was isolated from several samples of comparatively aged stages of the ripening process. It has the same relation as the preceding species on the storing of "shōyu", because it grows easily in "shōyu" and forms a thin film on the surface.

1. FORM AND SIZE. Cells are mainly globular, and $2.5-4.5\mu$ in diameter, but rarely $8-10\mu$. The gigantic cells which exhibit globular forms (measuring up to 20μ in size) are regularly found in the old culture. Further, this species is easily distinguished from the preceding species by forming extremely thick-skinned cells in the ring vegetations. Cell contents show none of the special peculiarities. Very old cells contain a single large fatty globule in the plasma.

2. GROWTH. The covering on "koji"-extract agar is almost the same as that of *Torula* α . This species develops well even at low temperature and strongly liquifies gelatine. It grows and produces a greyish white thin film in "koji"-extract, but the ring assumes a thicker, somewhat mucinous character, accompanied by some sediments in the course of further development. Growth in this medium is superior to that in wort or hopped wort. When the inoculated liquid media were kept for long periods the liquid became lemon-yellow. *Fermentation phenomena were not observed at all in any saccharine media.*

3. This species ferments neither dextrose, galactose, mannose, maltose, saccharose nor raffinose, but grows well in yeast water which contains one of these sugars.

4. This species also reproduces by budding only.

G. MYCODERMA α .

The authors isolated two species of *Mycoderma* from some "shōyu-koji." But there are only slight differences between both species, hence, they may be described as one and the same species.

1. FORM AND SIZE. Cell form is not constant. In the film filamental cells predominate, but sausage shapes or pastorianus forms are often found. Neither are globular cells rare. Cell content is pale with large vacuole, and

from one to three small fatty globules are seen in every cell. Cell dimensions vary considerably. The following numbers are given from numerous mensurations with cells which developed in "koji"-extract at 25° for 10 days.

5 × 3.3μ	8 × 4μ	11 × 5μ	23 × 3.3μ
6.5 × 3μ	6—5μ	15 × 3.3μ	20 × 4μ

2. GROWTH. Solid culture: On "koji"-gelatine plate it forms a dirty white, flat colony with some folds in the centre and mycerial growth at the margin. Surface culture on "koji"-extract agar shows a greyish white mesenteric coating with filmy radiated margin. Fluid culture: In the course of 24 hours it already forms a greyish white thin film on the surface of "koji"-extract (at 25°). The liquid becomes gradually cloudy. After 3 days the film becomes somewhat dried and wrinkled. A moderate evolution of carbonic acid gas is easily observed, accompanying the production of ester flavour. The cloudiness disappears and the film becomes thicker and whitish yellow after the lapse of half a month.

3. BEHAVIOUR TOWARDS SUGARS. This fungus ferments dextrose, mannose, saccharose (?), but not galactose, maltose, lactose, raffinose, inulin and α -methylglucosid.

4. FORMATION OF SPORE. Sporulated cells have never been seen in any spore culture in spite of many trials.

5. CONDITIONS OF TEMPERATURE: Heating to 60°C for 25 minutes is not sufficient to destroy this yeast, however, it is completely destroyed by heating at 60° for 30 minutes. Optimum temperature for the growth lies between 30° and 31°C.

Summary of Part First.

1. It is a very interesting fact that five different species of *Zygosaccharomyces* were isolated from "shōyu-moromi."

2. *Zygosaccharomyces major* was mostly isolated from samples of mature stage, while *Zygosaccharomyces soja* was obtained from the samples of young stages of the ripening process. There is no doubt that these two *Zygosaccharomyces* play an important rôle in the ripening of "shōyu."

3. *Zygosaccharomyces japonicus* and *Zygosaccharomyces salinus* easily produce greyish white, crape-paper-like films even on a concentrated "shōyu," which could no more grow any other kinds of film-forming yeast. Moreover, these two yeasts form a large number of sporulated cells with easiness. They are therefore most dangerous to the storing of "shōyu."

4. It is probable that specific differentiations of "shōyu" yeasts, which have been studied by several authors, have not agreed with one another owing to their sporulating difficulties.

5. If it be desired to observe the spore formation of "shōyu" yeast it should be undertaken in the following way:

The yeast is sowed in a sterilised test tube which contains a quantity of "shōyu," diluted with water to make its salt-content 5%, and the culture is laid at 28° C. for the first 3 days and then immediately kept at 20°-25° C. for 7-15 days.

6. A special species of *Monilia* indigenous to "shōyu-moromi" was isolated. The characteristics of this species taught us many instructive facts, and amongst them may be mentioned the faculty of propagation of the fungus in the pabulum containing 18-22% of NaCl, or at a temperature as high as 40°C. The decolorization of "shōyu" by this fungus is a property not to be overlooked; because the *dense colorization* of "shōyu" is in general one of the important factors to qualify "shoyu."

7. Among *Torula* two species α and β , may be mentioned; the former fermenting glucose, laevulose, maltose and saccharose, while the latter lacks the fermenting property. Both species flourish on the surface of "shoyu", which is decolorized and alters to a marked alkaline reaction.

8. *Mycoderma* isolated from "shōyu-koji" causes *mannose* to ferment, but it is of minor importance in the manufacture of "shōyu", except in imparting a *desirable aroma* to "shōyu-koji."

PART II. THE FERMENTATION PRODUCTS.

In the research for the fermentation products, all varieties, except two, of "shōyu"-yeast isolated were infected to "koji"-extract (12°B) and held at

23–25°C, being shaken twice every day, and after 16 days analyses were made.

I. ZYGOSACCHAROMYCES MAJOR, NOV. SPEC.

The analytical data from the culture of fifteen varieties are as tabulated below :

Grams in 100 c.c.										
Yeast variety.	Total ester. ¹	Volat. ester. ²	Alcohol vol. %	Non-v. acids ³	Vol. acids ⁴	Amino acids ^{4a} assimilated	Coeff. of assi- milation.	Try- pto- hyd. oil. ⁵	Fusel	Remarks. ⁶
15. a....	0.165	0.117	4.18	0.014	0.013	0.138	42.88	±	—	++ { Probab. Hry butylalcohol.
15. b....	0.124	0.066	7.0?	0.023	0.001	0.117	36.41	—	/	+ The same.
8. I....	0.162	0.059	7.0?	0.019	0.014	0.081	25.14	+	—	++ /
{ 23.....	0.129	0.053	6.400	0.014	0.015	0.128	40.35	—	Trace.	+ { Probab. Hry butylalcohol.
	22.....	0.132	0.077	6.400	0.018	0.004	0.128	40.36	—	—
19.....	0.053	.00	5.530	0.011	0.013	0.115	35.89	—	—	+ Same.
20.....	0.199	0.047	4.880	0.017	0.006	0.142	44.14	—	—	++ Same.
11.....	0.118	0.076	4.880	0.005	0.023	0.117	36.86	±	/	++ { Probab. Iso-propylalcohol.
32.....	0.383	0.142	3.420	0.014	0.011	0.159	49.83	—	—	++ { Probab. Hry butylalcohol.
10.....	0.173	0.076	3.420	0.011	0.019	0.123	38.43	—	—	++ { Probab. Iso-propylalcohol.
H. T....	/	/	3.420	0.013	0.015	/	/	/	Trace.	++ Same.
N. C....	0.324	0.177	2.720	0.019	0.007	0.049	15.5	+	—	+ /

[+ denotes presence, — absence and ± ambiguous. Original solution contains 0.32g amino-acids, 0.0218 g total acids in 100 c.c.]

The varieties No. 22 and 28 may be identical but we will describe them separately until some detailed observations will settle the question. The variety No. 32 has the largest assimilability of all varieties observed, for amino-

1. Total ester is calculated as ethylsuccinate.
2. Volatile ester is calculated as ethylacetate.
3. Calculated as succinic acid.
4. Calculated as acetic acid.
- 4a. Coefficient was found by comparing assimilated aminoacids to those of the original solution, the latter assumed as 100.
5. The reagent used is vanillin-sulphuric acid (TAKAHASHI's method). Refer to "Journ. of the College of Agric. Tokyo Imp. Univ. Vol. V. No. 2.
6. The examination of higher alcohols was made separately after Mullekin's direction: Mullekin's Identification of Pure Organic Com. Vol. I.

acids e.g. the coefficient of assimilation is 49.83. Generally, the variety which produces somewhat large quantities of alcohol has the tendency to form relatively smaller quantities of ester.

II. ZYGOSACCHAROMYCES SOJA, NOV. SPEC.

Yeast var.....	8	23	10	a
(Grams in 100 c.c. of the sample).				
Total ester.....	0.604	0.162	0.221	0.177
Volatile ester.....	0.313	0.088	0.077	0.094
Alcohol. vol. %.....	7.18?	5.53	3.42	1.16
Non-Vol. acids } formed.	0.0185	0.0136	0.0136	0.0285
Volatile acids } formed	0.0108	0.0120	0.0150	/
Amino acids:—				
Assimilated.	0.1165	0.1548	0.1548	0.0536
Coeff. of assimil.	36.33	48.39	48.39	16.70
Tryptophane.	+	±	+	+
Aldehyd.	Trace.	Trace.	Not found.	Not found.
Fusel oil.....	Present.	/	Present.	Present.
Remarks.....	{ Probably mainly Iso- propylalcoh.	{ Probably Iso Butyl- alcohol.	{ Probably mainly Iso- propylalcoh.	{ Probably Iso Butyl- alcohol.

Thus, the variety No. 8. shows parallelism in the production of alcohol and esters, and such phenomenon was not met with in the former group.

MASAO KURIYAMA further studied the varieties No. 8 and 23 with the cultures in “koji”-extract or “shōyu-koji”-extract by the addition of NaCl in varying quantities. The cultures were held at 25°C and the analytical results after the complete fermentation of the extracts were found.

Alcohol. vol. %.

Yeast Variety	Culture media.	“koji”-extract.	“koji”-extract +5% NaCl.	“shōyu-koji”-ext. +10% NaCl.	“shōyu-koji”-ext. +15% NaCl.
8.....		3.49	3.28	1.75	1.29
23.....		2.73	2.58	0.67	0.67

Total acids (as succinic acid. g. in 100 c.c.)				
8.....	0.1146	0.0764	0.0425	0.0319
23.....	0.1273	0.1018	0.0530	0.0637

Non volatile acids (g. in 100 c.c. as succinic acid).				
8... ..	0.1019	0.0764	0.0425	0.0319
23.....	0.1146	0.0764	0.0531	0.0637

Volatile acids (g. in 100 c.c. as acetic acid).				
8.....	0.0129	0	0.0	0.0
23.....	0.0129	0.0259	0.0	0.0

Coefficient of assimilation of amino-acids. ⁷				
8.....	26.26	27.67	19.85	21.95
23.....	40.41	42.85	20.56	25.75

Fusel oil% (TAKAHASHI's method :—anisaldehyd and sulphuric acid).				
8.....	0.030	0.03	0.03	0.025 less
23.....	0.030	0.03	0.025	0.025 less

Ester number.				
8.....	2.4	1.6	2.0	2.8
23.....	2.8	2.8	1.6	2.0

The depressing influence of natriumchloride on the fermentation of “shōyu”-yeast is manifest from the above data, confirming TORAZŌ NISHIMURA's⁸ and SANYA MORI's observation on the same subject. The quantity of the organic acids formed is generally, but with exceptions, decreased in solutions which contain NaCl. As regards the ester formation a kind of contrary phenomenon is observed as it increases in quantity with higher amounts of NaCl.

The assimilability of the amino acids differs not only according to the varieties but also according to the culture media. So, “koji”-extract containing 5% of NaCl gives a higher assimilability than common “koji”-extract

7. Amino-acids in the original “shōyukoji”-extract were 0.359 gr. and that of “koji”-extract 0.254 gr. in 100 c.c.
8. Naikokuzeisan (Japanese). 1909.

devoid of common salt, and “shōyu-koji”-extract which contains 15% of NaCl expresses a higher assimilation coefficient, in a certain degree, than extract with 10% of NaCl. A noteworthy fact is the special higher assimilability of amino acids in “koji”-extract (5% NaCl) than in “shōyu-koji”-extract (10% NaCl). In the former case the coefficients of the assimilation of amino acids are 27.67 and 42.84, while in the latter case they are 19.85 and 2.55. If such property of the yeast holds good in the practical manufacture of “shōyu,” there must be a large difference between the finished “shōyu”, which is prepared simply from “shōyu”-koji, and that which is admixed partly with “koji” from boiled rice.⁹

III. ZYGOSACCHAROMYCES SALSUS, NOV. SPEC.

		grams in 100 c.c.						
Yeast variety.	Alcohol vol. %.	Non. vol. acids, formed.	Volatile acids, formed.	Aminoacids, formed, assimilated.	Coef. of as- simil. of aminoacids.	Tryp- phan.	Aldehyd.	Fusel oil.
E	4.18	0.0249	0.0071	0.1339	44.89	--	Trace.	present. ¹⁰
F	4.88	0.0199	0.0054	0.0748	23.30	±	not found	present.

The difference of the assimilability of amino-acids of E and F varieties is a noticeable fact among other things.

IV. ASPOROGENIC SPECIES OF ZYGOSACCHAROMYCES (?).

Yeast variety.	Alcohol vol. %	Esters total.	Volatile esters.	grams in 100 c.c.			Coeff. of as-similation of amino-acids.	Tryp-phan.	Alde- hyde.	Fusel oil.
				Non-Vol. acids formed.	Volatile acids formed.	Amino- acids as-simulated.				
18. ₁	6.40	0.174	0.106	0.0161	0.0120	0.145	45.33	—	not found.	present. ¹¹
27	6.40	0.119	0.065	0.0118	0.0197	0.111	34.64	—	not found.	present. ¹²
P + a.d. ...	5.53	/	/	0.0132	0.0086	0.077	24.00	+	not found.	present. ¹³

Both varieties 18.¹ and 27 have many similarities in their products, except in the quantity of the volatile esters and the coefficient of the assimilation of amino-acids, while the variety p_{+a.d.} differs from both of them in

9. In some “shōyu” factories rice-“koji” is also added as a raw material.
10 and 13. Probably isopropyl alcohol is the chief constituent beside amylalcohol.
11 and 12. Hry butylalcohol is the chief constituent beside amylalcohol.

regard to the volatile acid produced and the presence of isopropylalcohol in fusel oil, also by a lesser assimilability of amino-acids.

MASAO KURIYAMA's observation on variety 27 shows :—

Culture.	Culture media.	" koji "- extract.	" koji "-ext. + 5% NaCl.	" shōyukoji "- ext. + 19% NaCl.	" shōyukoji "- ext. + 15% NaCl.
Alcohol vol. %.....		3.56	3.35	1.48	1.34
Total acids					
grams in 100 c.c.		0.0636	0.0764	0.0531	0.0531
Non-V. acids ,, ...		0.0255	0.0637	0.0531	0.0425
Volatile acids ,, ...		0.0388	0.0129	0.000	0.0108
Coeff. of the assim.					
of amino-acids.		18.867	14.308	18.997	17.307
Fusel oil vol. %.....		0.03	0.025	0.025	0.025
Ester number		1.6	2.0	2.8	4.0

The retarding action of NaCl to the alcoholic fermentation is also to be met with in this variety as in the case of *Zygosaccharomyces soja*, but the formation of the esters is rather accelerated by the salt, as we have mentioned already.

The assimilation of the amino-acids is retarded, in contrast to the case of *Zygosacch. soja*, in proportion to the concentration of NaCl added.

V. ZYGOSACCHAROMYCES JAPONICUS, SAITO.

The " koji "-extract was the same as in the other case, but the conditions of the culture differ inasmuch as it was held at 32°C during nine days. The analytical results of the fermentation products are :—

Alcohol. vol. %.	Total acids, formed.	Non-V. acids, formed.	Volat. acids, formed.	Amino-acids, assimilated.	Coeff. of assimil. of amino-acids.
2.72	————	————	————	0.132	36

[In the orig. solution amino-acids were found to be 0.369 %].

VI. SPECIES OF MYCODERMA, ISOLATED FROM "SHÖYU-KOJI."

Grms. in 100 c.c.										
Yeast variety.	Alcohol vol. %	Total esters.	Volatile esters.	Non-Vol. acids, formed.	Volatile acids, formed.	Amino-acids, assimilated.	Coeff. of assimil. of amino-acids.	Tryptophan.	Aldehyde.	Fusel oil.
B.....	3.42	0.044	0.029	0.0043	0.0010	0.111	34.64	±	not present.	present.
C.....	3.42	0.173	0.034	0.0014	0.0034	0.069	21.60	—	present.	present. ¹⁴

The esters formed by both varieties have a special good flavour, like amylic or butylic ester, and further investigations with this species are likely to give good results.

VII. PICHIA ALCOHOLOPHILA, KLÖCKER. VAR.

For this fungus the "koji"-extract used as a culture medium indicated 14° of Balling's saccharometer, but the conditions during the cultivation were quite the same as in the case of the other fungi.

Alcohol vol. %	Total esters.	Volat. esters.	Total acids,* formed.	Volatile acids, formed.	Non-V. acids, formed.	Amino-† acid, assimilated.	Coeff. of assimil. of amino-acids.	Fusel oil.
0.421	0.2221	0.0176	0.078	0.0144	0.0545	0.1150	79.31	present.

This fungus must be included in the genus *Pichia* on account of its morphological identifications, whereas alcohol was found, though in traces, in the culture, as Klöcker's‡ recent report describes.

VIII. TORULA SPEC.

The yeast of this species was found in "shōyu-koji" and "shōyu"-moromi of various stages, young and old, in almost all samples we have observed, and the products of the fermentation are shown below:—

14. Isopropyl-alcohol is detected in this fusel oil.

† Amino-acids in the original "koji"-extract was 0.150% as glycocoll.

* The contents in the original solution amount to 0.0405% as succinic acid.

‡ Compt. Rend. d. Carles. 1913.

Grms. in 100 c.c.											
Yeast variety.	Alcohol. vol. %	Total esters.	Volat. esters.	Non-Vol. acids, formed.	Volat. acids, formed.	Amino-acids, assimilated.	Coef of as-simil. of amino-acids.	Trypto-phan.	Alde-hyd.	Fusel oil.	Origin.
{ 14...	3.42	0.177	Absent.	0.0077	0.0037	0.1281	39.98	—	—	?	{ Mash 9 months old.
{ 1...	3.42	0.243	„	0.0090	0.0053	0.0650	23.20	—	—	+	{ “shōyu-koji.”
{ 31...	3.42	0.265	0.1357	0.0090	0.0168	0.1186	37.01	—	—	±	{ Mash 10 months old.
{ 35...	2.72	0.236	0.0165	0.0096	0.0115	0.0298	8.10	—	—		{ Mash 17 months old.
{ 4...	2.72	0.140	0.0767	0.0048	0.0045	0.1300	40.58	—	—	+ ¹⁵	{ Mash 4 months old.
{ g...	2.72	/	/	0.0220	0.0060	0.0920	25.57	+	+	+	{ Mash 10 months old.
j. ...	1.06	/	0.0184	0.0117	0.0091	0.0310	9.30	+	—	Trace.	
p+a.4. Abs.		0.184	0.0177	0.0096	0.0017	0.0371	11.20	+	—	—	/
{ 21...	Trace.	0.237	0.0767	0.0015	0.0111	0.0882	27.52	—	Trace.	+ ¹⁶	{ Mash 8 months old.
{ 12...	Trace.	0.147	0.1062	0.0062	0.0222	0.1015	31.67	—	—	—	{ Mash 6 months old.
p+a. abs.		0.590	0.3186	0.0155	0.0130	0.0920	25.57	+	—	—	
37...	—	—	—	—	—	—	—		+	+	{ Mash 17 months old.

The quantities of alcohol produced vary according to the difference of the varieties, i.e. certain varieties produce 3.42, others 2.72 or 1.06, the remaining varieties only traces, or are incapable of forming alcohol. The genuine *Torula* after PASTEUR is incapable of causing alcoholic fermentation, and in general the acid production is rather less than its destruction, and such phenomena were observed in our yeast. But the decomposition of the acids in the pabulum in our case is found only in the non-volatile acids, but the formation of the volatile acids ensues as in the ananas *Torula* of KAYSER, BRETTANOMYCES or WEIGMANN'S *Torula*, and many others.¹⁸ The ester formation of *Torula* species is not unusual, so we omit further elucidation. The assimilation of the amino-acids is quite indifferent to the productivity of the alcohol, and as genuine *Torula* shows 31.67 as the coefficient of assimilability there is no trace of the formation of alcohol.

M. KURIYAMA'S observations on the variety 35 are of some interest compared with other varieties.

15. Isopropyl-alcohol is present in it beside amyl-alcohol.

16 and 17. Secondary butylalcohol is present beside amyl-alcohol.

18. The decrease of the acids in the pabulum is observed also in wine yeast. (Ventre. comp. rend. t. 157. No. 2. p. 154. 1913.)

Products.	Culture media.	"koji"- extract.	"koji"-ext. + 5% NaCl. ext.	"shōyu-koji"- ext. + 10% NaCl. ext.	"shōyu-koji". + 15% NaCl.
Alcohol vol. %.....		3.21	2.79	1.54	1.54
Total acids (grams in 100 c.c.)....		0.0891	0.1400	0.0849	0.0637
Non-v. acids. ,, ...		0.0764	0.1146	0.0743	0.0637
Volatile acids. ,, ...		0.0129	0.0259	0.0108	0.00
Amino-acids assimil.		0.0423	0.0484	0.0622	0.0682
Coeff. of the assimilation of amino-acids. ,, ...		16.617	19.025	17.307	18.997
Fusel oil. vol. %.....		0.025	0.025 less.	0.025 less.	0.01
Ester number.		1.6	2.4	2.4	2.0

The retarding influence of NaCl on alcoholic fermentation is evidently observed too in this variety.

The assimilability²¹ of the amino-acids is accelerated by the addition of salt, and especially so when "koji"-extract of boiled rice is used.

IX. MONILIA SPECIES.

This *Monilia* was isolated from "shōyu"-mash three months after the mixing of the raw materials, i.e. from very young "shōyu"-mash, and in many respects, we have reason to believe that this species is the same which was isolated by T. NISHIMURA¹⁹ from "shōyu-koji."

Grams in 100 c. c.								
Alcohol.	Total esters.	Volatile esters.	Non-Vol. acids, formed.	Volatile acids, formed.	Amino-acids, assimilated.	Coeff. of assimil. of amino-acids.	Trypto-phan.	Fusel oil.
Trace.....	0.165	0.236	0.0049	0.0175	0.0634	19.80	±	not found. present. ²⁰

X. OIDIUM.

Grams in 100 c.c.								
Fungus variety.	Alcohol vol. %.	Total esters.	Volatile esters.	Non-vol. acids, formed.	Volatile acids, formed.	Amino-acids, assimilated.	Coeff. of assimil. of amino-acids.	Trypto-phan.
29. C...	3.42	0.1843	0.0118	0.0107	0.0056	0.1224	38.19	-
G.	3.42	0.295	0.0159	0.0110	0.0206	0.0806	25.14	+
								not found. Trace.

21. The coefficient of the assimilation of amino-acids in KURIYAMA's experiment is about double that in our case, but in his case the acids originally present were only 0.255 gr. though in our case it was 0.320 gr. in 100 c.c. The quantities actually assimilated in both cases differ in a minute degree.

19. "Naikokuzeiisan." (Japanese).

20. Isopropyl-alcohol is present in it beside amyl-alcohol.

The formation of alcohol in this case is a noteworthy fact, for the largest quantities of alcohol ever observed from *Oidium* species are 1.2% in three months culture, by BREFFELD.

Summaries of Part Second.

1. All the varieties of *Zygosaccharamyces*, *Torula*, *Monilia*, *Mycoderma* and *Pichia* isolated from "shōyu"-mash or "shōyu-koji" assimilate amino-acids from their pabulum, in a less degree than saké yeast, *Saccharomyces saké*. The coefficient²² of the assimilation of amino-acids of saké yeast attains in some varieties above 83, while in the case of "shōyu"-yeast it hardly reaches 50, and there is no co-relation between the difference of the species of yeast and their assimilation coefficient of amino-acids.

It is out of question that the occurrence of such yeasts as assimilate amino-acids in lesser quantities is very favourable to the promotion of the quality of "shōyu," if the assertion that the quality of amino-acids has certain relations to the quality of "shōyu," as observed by U. SUZUKI²³ and others, is correct. In this respect it must be borne in mind that the solution of the varieties of "shōyu" yeast is of great importance in practice. Moreover, the retarding influence of NaCl on the assimilability of amino-acids is different according to the different varieties, and this fact induces us to change the time of adding common salt to the mash in accordance with the difference of the variety of yeast.

2. We are not bound to explain the evident fact that there is a definite relation between the decomposition of glutamic acid and the production of succinic acid during alcoholic fermentation. So, we have arranged, side by side, the quantities of amino-acids assimilated and that of non-volatile acid formed as follows:—

	Zygo. major. 32.	Zygo. soja. 23.	Zygo. soja. v. 10.	Zygo. ? 18. 1.
Coeff. of ass. of amino- ac.....	49.83	48.39	48.39	45.33
Non-volatile acids.....	0.0145	0.0139	0.0139	0.0163

22. As already explained, the coefficient was found by comparing amino-acids assimilated to the original ones, which were taken as 100 for convenience.

23. The Journal of the Tokyo Chemical Society. Vol. 31, No. 7.

	Zygo. major. 20.	Zygo. major. 15. a.	Torula. 4.	Zygo. salsus. 1.
Coeff. of ass. of amino-acids...	44.14	42.88	40.58	44.89
Non-v. acids formed.	0.0169	0.0127	0.0049	0.0253
	Zygo. major. 28.	Zygo. major. 22.	Torula. 14.	Zygo. major. N. 10.
Coeff. of ass. of amino-acids...	40.36	40.36	39.98	38.42
Non-v. acids formed.....	0.0145	0.0181	0.0079	0.0109
	Torula 31.	Zygo. major 11.	Zygo. soja. 8.	Zygo. major. 15. b.
Coeff. of ass. of amino-acids...	37.01	36.86	36.33	36.41
Non-v. acids formed.....	0.0091	0.0055	0.0187	0.0229
	Zygo. major. 19. 2.	Zygo ? 27	Mycoderma. B.	Torula. 12.
Coeff. of ass. of amino-acids...	35.89	34.64	34.64	31.67
Non-v. acids formed.....	0.0109	0.0027	0.0043	-0.0077
	Torula. p+a. 1.	Torula. g.	Torula. 21.	Zygo. major. 8. 1.
Coeff. of ass. of amino-acids...	25.57	25.57	25.14	27.52
Non-v. acids formed.....	0.0157	0.0223	-0.0017	0.0157
	Zygo. p+a. d.	Zygo. salsus. F.	Torula. 1.	Mycoderma. C.
Coeff. of ass. of amino-acids...	24.0	23.30	23.20	21.60
Non-v. acids formed.....	0.0133	0.0211	0.0031	0.0013
	Monilia. K.	Zygo. soja. a.	Zygo. major. II. C.	Torula. p+a. b.
Coeff. of ass. of amino-acids...	19.80	16.70	15.50	11.20
Non-v. acids formed.....	0.0013	0.0289	0.0199	0.0097
	Torula j.	Torula 35.		
Coeff. of ass. of amino- acids.	9.3	8.10		
Non-v. acids formed.....	0.0091	0.0097		

The diversity or irregularity between the varieties of "shöyu" yeast in the production of the non-volatile acids and the assimilability of amino-acids is shown very clearly in the table. It is highly probable that such diversity must be attributed to the varied assimilabilities of each variety for glutamic acid. So, if we take the five highest coefficients of the assimilation of amino-

acids in the above table, we will find the average to be ca. 46 or 47, corresponding to the 0.014% or 0.015% of non-volatile acids formed. From these data we can recalculate for the coefficient 35, 25, and 15, the non-volatile acids as 0.0114, 0.0082, 0.0049 in 100 c. c. The instances of overproduction of non-volatile acids beyond these figures calculated, are found in *Zygosaccharomyces salsa*, *E. Zygo. major* 15. b, *Torula* g, *Zygo. p+a. d.*, and *Zygo. soja* a, the reverse cases are to be met with in the figures of *Torula* 4, 12, 14, 31, g, *Zygo. major* 11 and others.

The former instances mostly those varieties which especially assimilate glutamic acid in larger quantities, leaving in the pabulum the other forms of amino-acids, and *vice versa* in the latter instances.

3. The formation of the esters during fermentation is depressed by the addition of NaCl in some varieties or species of "shōyu"-yeast, but the reverse is observable in the other varieties.

4. Certain species of the flora appear in the special phase of the fermentation of "shōyu"-mash, e. g. *Monilia* was found distinctively in the very young stage and *Mycoderma* varieties which, present in "shōyu-koji" almost constantly, have not till now been found in the fermenting mash. Moreover, hat shaped spores of *Willia anomala* are observed very often under the microscope in "shoyu"-mash, but the isolation of the fungus from the mash was not successful. The *Torula* species are distributed in all the stages of the mash, from young to old, while *Zygosacch. major* occurs most frequently in the old mash.

5. In the practical application of "shōyu"-yeast we are obliged to prepare at least two varieties of the yeast, inasmuch as we are not able at present to isolate a variety which is perfect from all points of view.

General Conclusions.

1. The occurrence of *Zygosaccharomyces* in "shōyu"-mash furnishes us with a very interesting field for future researches in microbiology. The application of pure cultures of *Zygo. major* and *Zygo. soja* must naturally result in the future improvement of our "shōyu." The two species of *Zygosaccharomyces* i.e. *japonicus* and *salsus*, and the species of *Torula* or *Monilia* must be

regarded as harmful or damaging fungi for "shōyu", but their common property of decolorizing "shōyu" may sometimes be utilized for the preparation of the colorless or "shiro-shōyu", by taking the precaution of lessening the deteriorating effect of the fungus. The *Mycoderma* species in "shōyu-koji" will be more appreciated in future on account of its energetic generation of a pleasant aroma.

2. The degree of the fermentation and the decomposition of the raw materials differ in noticeable amounts, so we must use special precautions in selecting the varieties of *Zygosaccharomyces* in this regard.

In concluding, the authors' thanks are due to Mr. M. SATŌ, assistant of our college, in assisting them in the analysis of the materials during the course of the researches.

Addendum.

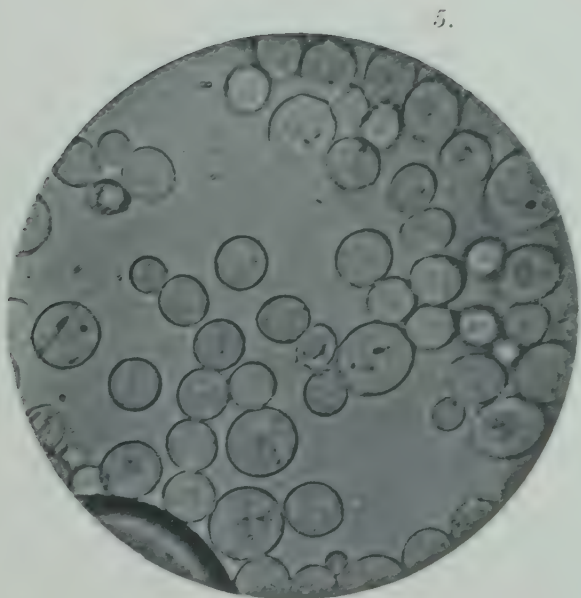
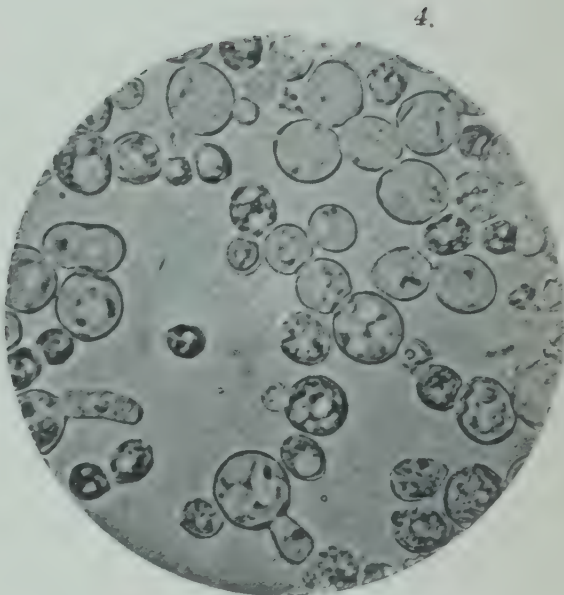
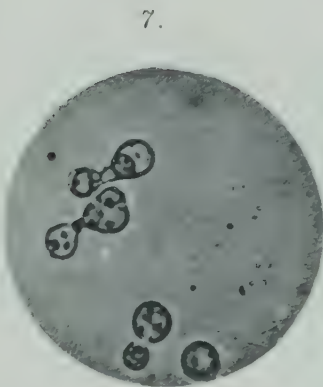
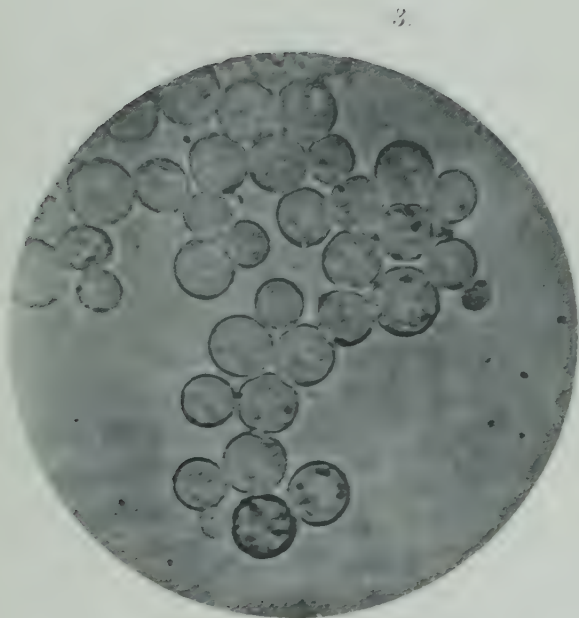
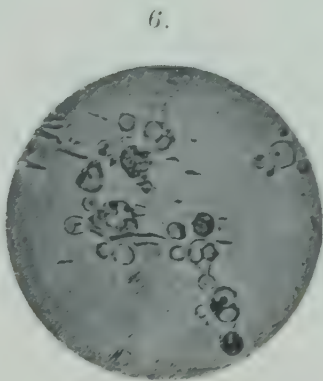
Recently G. KITA²³ published an article on the asporulation of "shōyu"-yeasts. As he merely stated, without going into details, that he tried vainly our diluted "shōyu"-method to observe the spore formation of his four yeasts, we cannot find any data to compare his yeasts with our *Zygosaccharomyces*. Moreover, we asked him to give us the cultures of his yeasts, but were unable to obtain these, as he had not preserved them.

23. Cent. f. Bak. II. Abt. XLI. 364.

EXPLANATION OF PLATE.

PLATE XV.

- | | | |
|---------|---|--|
| Fig. 1. | <i>Zygosaccharomyces major</i> ; | ¹⁵⁰⁰ / ₁ , after 2 days in koji-extract at 30°C. |
| Fig. 2. | <i>Zygosaccharomyces soja</i> ; | ditto. |
| Fig. 3. | Asporogenic species of <i>Zygosaccharomyces</i> ; | ditto. |
| Fig. 4. | <i>Zygosaccharomyces japonicus</i> ; | ditto. |
| Fig. 5. | <i>Zygosaccharomyces salsus</i> ; | ditto. |
| Fig. 6. | <i>Zygosaccharomyces major</i> ; | sporulated cells in diluted "shōyu" culture. |
| Fig. 7. | <i>Zygosaccharomyces japonicus</i> ; | ditto. |
| Fig. 8. | <i>Zygosaccharomyces salsus</i> ; | ditto. |
| Fig. 9. | <i>Zygosaccharomyces soja</i> ; | ditto. |



Researches on "Hatsuchō-Miso."

BY

Tasuku Akaghi, Iwawo Nakajima and Kunihiro Tsugane.

"Hatsuchō-miso" is a special variety of "miso" chiefly manufactured in Mikawa Province. This variety of "miso" has only a limited consumption amongst the Japanese, on account of its comparatively high price. On its chemical composition, especially on the amino-acids, T. TAKAHASHI and G. ABE have reported already in 1908.¹

In 1911, Y. NISHIWAKI'S² article on *Oidium lupuli* which occurs frequently, especially in the warm season, in "koji" of "Hatsuchō-miso", was published, and his report stimulated G. KITA³ to apply this fungus to a quite different branch of industry, i.e. to the spiritus industry, as a saccharifying fungus. In preparing "Hatsuchō-miso," the well cleaned soy-bean is steeped⁴ in water and boiled for some six or seven hours. The boiled soy-bean is crushed in a special apparatus and then moulded in a round or cylindrical mass suitable to lay on the floor of the "koji"-chamber i.e. a chamber constructed for the preparation of "koji." After some 20-40 days, "koji" ripens⁵, hardened and covered with the spores of the mould. Then it is mixed with common salt and water, and is introduced into a fermenting vat covered by straw mats, weighted with a large number of stones, to ensure the materials ripening en masse. The ripening of "Hatsuchō-miso" is generally attained in from three to five years.

The Microbes found during the Preparation of "Koji."

I. MOULD FUNGI.

In investigating "koji" during its preparation we find several species of mould fungi as mentioned below:—

1. The Journal of the Tokyo Chemical Society, Japan, Vol. 29, No. 2, Feb. 1908.
2. "Jōzōzasshi" (Japanese) No. 434. p. 17. 1911.
3. Deutsch. Essigind. Dec. 1813.
4. By the steeping process the volume of the soy-bean increases by 30-50% of the mass.
5. Three parts of "koji" are obtainable, in round number, from two parts of the soy-bean. [Jour. Coll. Agric., Vol. V, No. 3, 1915.]

1. *Aspergillus Oryzae*, includes two varieties.
2. *Asperigillus Oryzae* var. *fulvus*, T. Yamamoto.⁶
3. *Sterigmatocystis* with blue spores.
4. *Aspergillus niger*.
5. *Penicillium luteum*.
6. *Penicillium glaucum*.
7. *Mucor pyriformis*.
8. *Rhizopus* sp.
9. *Rhizopus nigricans*.
10. *Oidium lupuli*.

These species of mould fungi are found almost in every matured "koji", but the species predominating during the stages of preparation differ more or less in accordance with the season in which the preparation is carried on. During winter, when the atmospheric temperature is low, the temperature of the "koji"-chamber is affected; so we usually find species of *Mucor*, *Rhizopus* and *Penicillium* in the first stage of the "koji"-preparation, and in the second stage *Penicillium* and *Rhizophus*, and at the end of the process the varieties of *Aspergillus Oryzae* and in a small part of the preparation *Oidium lupuli*. On the other hand, in the preparation during the warmer season, in spring and autumn, *Aspergillus Oryzae* and *Oidium lupuli* are predominating growths.

The degree of decomposition of the protein-matter in the boiled soy-beans by these fungi must not be overlooked; for it may be regarded in a certain degree as a criterion of the change which occurs during the preparation of "koji." For the purpose the washed soy-beans were steeped for one hour in water and were then subjected to discontinuous sterilization, once every day for three hours, four times in total, and after the infection with the spores of the fungi described, the cultures were held at 28°C, except the *Penicillium* culture, which was kept at 20°C, during seven days. The cultures, thus obtained, were extracted with boiling water three times, and in this extract, the soluble protein⁷, N⁸-subs. precipitated

6. T. Yamamoto. Jōzōshikenjohōkoku. No. 42. 1912.

7. STUTZER'S method.

8. After the removal of the protein matters by STUTZER'S method.

by phosphotungstic acid, amino-acids⁹, and ammonia¹⁰ were determined as the data below :

	Protein-N. in 100 grm. of "koji."	N. ppt. by pht. acid.	Formol-amino-acid. as glycocoll.	Ammonia-N.
	gr.	gr.	gr.	gr.
Control. i.e. boiled soy bean.	1.9815	0.0348	0.5668	0.0535
<i>Aspergillus Oryzae</i> 1.....	1.0274	0.7192	3.4008	0.7781
" " 2.....	0.6311	0.8073	3.8259	0.9994
" " var. <i>fulvus</i>	0.4990	—	4.4636	1.2065
<i>Oidium lupuli</i>	0.8219	0.7045	1.4819	0.2479
<i>Rhizopus</i> spec.....	0.8073	0.6605	2.6923	0.1499
<i>Rhizopus nigricans</i>	0.7632	0.4990	1.4819	0.4854
<i>Sterigmatocystis</i> spec.....	1.0128	0.7486	3.4008	0.4712
<i>Asper. niger</i>	0.4110	0.7926	5.5263	1.2851
<i>Penic. luteum</i>	0.6165	0.6605	1.0635	0.1964
<i>P. glaucum</i>	0.6311	0.4990	1.6300	1.1780

Thus the remaining soluble protein was found largest in the culture ("koji") of *Asp. Oryzae* 1. and least in the culture of *Asp. niger*, and generally the quantities found in the culture of all species show more or less decrease compared with that of the original soy-bean boiled. The nitrogenous substances precipitated by phosphotungstic acid increased as a result of the decomposition of the protein, and the amino-acids, too, show the same change, although the increase in quantity is variable according to the different species. As we have shown in the table, there are minute differences found in the quantities of the bases (ppte. of phosphotungstic acid) with regard to the increase by each species of the fungus; while in the increase of the amino-acids there are noticeable differences according to the species of the fungus concerned. The formation of ammonia occurs in every culture of the species in an analogous rate with the amino-acids.

The liquefying property of these fungi for gelatine has an interesting relationship to the quantities of ammonia formed by the species as the latter are almost parallel in regard to the degree of liquefaction. The test was made by infecting the "koji"-extract-gelatine with the species found

9. Formol method after SØRENSEN.

10. WURSTER's method.

(10cc. and in the inclined surface) and held at 17°C, which gave the following results:—

	After nine days.	After 14 days.	After 16 days.	After 20 days.	After 30 days.
<i>Aspergillus Oryzae</i> 1.....	—	—	+	++	++++
" " 2.....	++	++	+++	+++	+++++
" " var. <i>fulvus</i> ...	+	+	++	+++	+++++
<i>Oidium lupuli</i>	+	++	++	++	+++
<i>Rhizopus spec.</i>	—	—	—	+	+
<i>R. nigricans.</i>	—	—	+	++	++
<i>Sterigmatorystis spec.</i>	—	—	+	++++	+++++
<i>Aspergillus niger</i>	—	+	+++	++++	+++++
<i>Penicillium luteum.</i>	—	—	+	+	+++
<i>P. glaucum</i>	+	—	+	—	++++
<i>Mucor pyriformis</i>	—	—	—	+	+

[— denotes absence, + + + + + denotes liquefied 10cc. and + denotes trace.]

II. YEAST AND BACTERIA.

From “koji” three species of yeast were isolated; *Torula*, *Willia anomala* and *Mycoderma spec.* The species of *Torula* having a round or short elliptic cell, could not ferment glucose, galactose, saccharose, lactose and dextrin. Nor was there in “koji”-extract any sign of fermentation. *Willia anomala* behaves analogous to the varieties of the species described as the aging yeast of saké by T. TAKAHASHI¹¹ and H. SATO. It ferments glucose and very sparingly galactose but not cane-sugar, lactose and dextrine. *Mycoderma spec.* ferment glucose, galactose but not lactose and dextrin.

In regard to the species of bacteria found in “koji” the observation is too incomplete to describe definitely, but *Sarcina cervina* and *S. aurantiaca* and *Bacillus proteus fluorescens* and other motile and non-motile species of the bacillus were found. The above mentioned three species of bacteria develop in soy-bean-extract containing 13% of NaCl, and are distinguished from the other species isolated. Their peptonifying properties are relatively small in comparison with the fungi described.

11. Journ. of the College of Agric. Imp. Univ. of Tokyo. Vol. 1. No 3. p. 227-263.

The Chemical Changes occurring during the Ripening of
"Hatsuchō-miso."

From the foregoing descriptions of the species of fungi we have a thorough knowledge of the chemical changes which must have occurred during the preparation of "koji", i.e. the decomposition of carbohydrate and protein matters in the steamed soy-beans, giving rise to the accumulation of the decomposed products. In the sample of "koji" prepared by a manufacturer of "Hatsuchō-miso" we have observed 0.438% amino-acids, and 1.075% of glucose at the beginning of the preparation; while in the sample of the ripened "koji" they reached 1.615% of amino-acids and 3.846% of glucose in the fresh state of the sample.

A similar change must occur in the mash of "Hatsuchō-miso," although the retarding property of the common salt acts antagonistically to the decomposition of the raw materials. Besides, the behavior of the species indigenous to the mash must be especially mentioned.

A variety of *Oidium* develops well in the mash especially during the first 3 months after the mixing of the raw materials, and some species of *Sarcina* and *Bacillus*, forming some non-volatile acid (lactic acid?) and a trace of acetic acid, are found. They must not be overlooked in regard to the ripening of "Hatsuchō-miso," but we are not in a position at present to describe them in detail. The chemical changes occurring in the mash are shown in the following table.

	"Koji."	Young mash.†	Ripened‡ 11, or "Hatsuchō-miso."
Water	33.80	46.616	44.780
Dry matter.....	66.20	53.384	55.220
therein.			
Crude fiber.	—	5.6781	5.4099
Ether extract.	8.042	18.420	16.480
Sugars after inversion.....	11.10	11.04	10.02
therein { Glucose*.....	5.30	6.65	7.58
Glucose, originally present.....	5.807	4.39	2.44
Total acids as lactic acid.		0.0487	0.1189
Volatile acids as acetic acid	—	—	0.0038

Total nitrogen.....	————	6.525	6.604
Protein, crude.....	————	————	————
therein 1. Soluble {			
1. Pptd by basic Pb.—acetate.....	8.775	{ 0.989 (as N). 8.5750	{ 1.041 (as N). 6.5063
2. Stutzer's method.....	6.374	{ 0.852 (as N). 5.3250	{ 0.623 (as N). 3.8938
„ 2. Insoluble.....	————	{ 2.387 (as N). 17.30	{ 1.9265 (as N). 13.9671
Amino-acid as glycocoll.....	2.440	4.672	6.748
„ —N.....	0.455	0.8709	1.260
Ammonia—N.....	0.124	0.5114	0.5784
Ammonia.....	0.3014	1.2427	1.455
Albumose.—N.....	————	0.0981	0.1210
Peptone + Base—N, except Ammonia.....	————	0.5116	0.8037
Ash.....	————	————	18.9155
NaCl.....	————	————	15.744

* All reducing sugar after the inversion, except the glucose originally present in the mash.
† 15 days after the mixing of the raw materials.
‡ 20 months after the „ „ „ „ „

Summary.

Although our observations described above are mere preliminary work in regard to the scientific points of view, yet there are some interesting facts not to be overlooked. Our consumers of “Hatsuchō-miso” always notice its special flavor or aroma, and its taste, and its high commercial value is naturally dependent upon both these properties. The nature of the aroma is not explained at present, but the taste of it is ascribed to the water soluble matter beside protein-matters¹², so that a part of it at least must consist of the decomposed substances derived from protein-matters. In this regard we are obliged to consider the quantities of amino-acids in “koji,” “Hatsuchō-miso” and the pure culture of the mould fungus isolated from “koji.” In “koji” 2.44% (or 1.615% in the fresh state), in “miso” 6.748% (or 3.726%

12. Cf. T. TAKAHASHI's article. The Journal of the Tokyo Chemical Society, Japan. Vol. 29. No. 2. Feb. 1908.

in the fresh state) in dry matter and in the pure cultures of our fungi 3.4-5.52% or the like are found in the fresh state. As a very clear conclusion it follows that, if we prepare pure "koji" by selecting the most suitable fungi, the amino-acids in the mash can be easily increased, or sometimes the period of fermentation of the mash can be shortened, and the manufacturer will soon find his products improved and his invested capital turned over more quickly.

The Action of Takadiastase on the Digestive Power of the Healthy Animal.

BY

Shin Sawamura.

It is generally a practice in medicine to give digestive enzymes such as pepsin, takadiastase, etc. to patients suffering from dyspepsy, and it is believed that the digestive power of the patient is accelerated by this. Whether the digestive enzymes taken in together with food by healthy animals accelerate digestion is, as far as I know, not yet settled. Green fodder, such as grass, tubers, roots, etc. contains various enzymes, such as peptase, diastase, etc. as proved by SCHEUNERT and GRIMMER,¹ and TADOKORO². If the enzymes contained in food stuffs accelerate digestion in the animal body we must prefer green fodder to the dried. To decide this the author tried to find the effect of digestive enzymes given together with fodder upon the digestion in the healthy animal body.

In 1911 March 20 a Merino ram was put in a HENNEBERG's feeding box, to become accustomed to this way of living, and a ration consisting of 2 parts of hay; 1 part wheat bran and 1 part starch was given. The hay was of wild-grown cereal grass, and the starch was obtained from batatas. The ration was given at 8 A.M. and 4 P.M. in the following quantities:—

	Morning.	Evening.
Hay.....	240 gr.	120 gr.
Bran.	120	60
Starch.	120	60

The sheep was healthy and devoured the food completely, and his live weight increased from 36.2kg. to 37.5kg. during 5 days of feeding. On the 22nd the dung was stained with iodine and microscopically examined,

1. Zeitschrift für Physiol. Chemie B. 43. 1906).
2. Journal of Coll. Agriculture, Sapporo. Vol. V. II.
[Jour. Coll Agric., Vol. V, No. 3, 1915.]

by which many starch granules were found. On April 24th the dung bag and urine funnel were attached to the sheep, and after this the fodder given, and that left uneaten, and the dung were exactly weighed, and $\frac{1}{10}$ of the dung was dried at low temperature and kept for analysis. The fodder given was 1kg. at first and after a few days increased to 1.1kg. But as the sheep left much of it uneaten, it was again decreased to 1kg. At first 2 litres of water were given, but on the 26th as the sheep excreted pasty dung, less water was given, on which the dung became normal. The feeding experiment was continued to the 30th. The live weight and the quantity of fodder taken in, water drunk, feces and urine, were as follows:—

TABLE I.

Date.	April 24.	25	26	27	28	29	30	Total.
Live weight (kg.).....	37.0	—	—	—	34.7	35.0	35.0	—
Fodder (gr.).....	979.0	988.0	1039.0	1028.5	1022.0	859.0	858.0	6774.0
Water (c.c.).....	2350	2360	1150	1000	1350	1525	1160	10835
Feces fresh (gr.).....	1464	1224	1131	1162	995	855.5	1040	7871.5
„ (gr.).....	—	—	—	—	—	—	—	2250.0

On May 1 the dung bags were removed and the sheep were allowed to move freely in the boxes. Starch granules were observed in the dung also on this day. From this day on the same fodder as in the previous experiment with 0.5 gr. of takadiastase was given. The takadiastase used was manufactured by Parkes, Davis & Co. according to DR. TAKAMINE'S process, and it contained 9.3% of water and 8.36% of crude protein ($N \times 6.25$). Several starch granules were observed in the dung of the sheep when examined every day.

On May 8 the dung bags were fastened to the sheep and 1 – 1.1 kg. of the fodder above described with 0.5 gr. of takadiastase and 2 l of water were given every day. The sheep seemed at first a little inactive, but afterwards regained activity and showed very good appetite, and their live weight increased. The feeding continued to the 14th. The live weight and the quantity of fodder eaten, water drunk, feces and urine, were as follows:—

TABLE II.

Date.	May 8.	9	10	11	12	13	14	Total.
Live weight (kg.).....	34.6	35.0	35.5	37.0	37.0	37.3	37.3	—
Fodder (gr.).....	935	974	949.5	999	1096.5	1076	938	6968
Water (c.c.).....	1900	1890	1420	1350	900	1780	1775	11015
Eeces, fresh (gr.).	954	930	858	802	950	807	946	6247
„ air-dry (gr.).....	—	—	—	—	—	—	—	2235
Urine (c.c.).....	330	250	370	300	250	230	150	1880

In the chemical analysis of the fodder and feces, the water was estimated by drying the sample at 100°C. to the constant weight; crude protein by multiplying nitrogen found by KJELDAHL'S method with 6.25; crude fat by extracting the sample with ether; and crude fiber by Weende method. Glucose was estimated in the aqueous extract of the sample. The extract was heated with Fehling's solution, and the cuprous oxide produced was dissolved in HCl containing some chlorine. The solution was made alkaline with ammonia and titrated with KCN, and from the weight of CuO glucose was calculated. Starch was estimated as glucose by inverting it with HCl. The composition of the fodder and feces was as follows:—

TABLE III.

	Fodder.	Fodder remained.		Feces (air dry).	
		Period I.	Period II.	Period I.	Period II.
Water.....	14.325%	9.745%	10.885%	8.735%	8.450%
Crude protein.....	9.462	9.462	9.462	12.388	12.388
Crude fat.....	3.140	2.535	3.265	3.740	3.585
Crude fiber.....	21.960	5.227	6.410	29.760	29.925
Pentosan.....	15.223	4.540	10.528	20.000	22.410
N. free extract.....	46.305	69.339	65.766	34.263	34.497
Starch.....	30.809	64.125	44.775	8.415	8.483
Glucose.....	3.768	1.250	1.250	0.600	0.525
Crude ash.....	4.808	3.692	4.212	11.114	11.155

The digested nutrients in Periods I and II, calculated from the above data were as follows:—

TABLE IV.

PERIOD I.

	Given. (gr.)	Remained. (gr.)	Eaten (gr.)	In feces. (gr.)	Digested. (gr.)	Dig. co- efficient %.
Dry matter.....	6339.950	564.996	5774.954	2053.463	3721.491	64.44
Crude protein.....	700.118	59.232	640.956	278.720	362.226	56.51
Crude fat.	232.360	15.869	216.491	84.150	132.341	61.13
Crude fiber.....	1625.040	32.721	1592.319	669.600	922.719	57.95
Pentosan	1126.502	28.420	1098.082	450.000	648.082	59.92
N. free extract.	3426.570	434.062	2992.508	770.918	2221.590	74.24
Starch.	2279.866	401.423	1878.443	189.338	1689.105	89.92
Glucose.	278.872	7.825	271.007	13.500	257.507	95.02
Crude ash.	355.792	23.112	232.680	250.065	82.615	24.83
Nutritive ratio.....	—	—	—	—	—	9.57

PERIOD II.

	Given. (gr.)	Remained. (gr.)	Eaten. (gr.)	In feces. (gr.)	Digested. (gr.)	Dig. co- efficient %.
Dry matter.....	6425.625	474.092	5951.533	2046.143	3905.390	65.62
Crude protein.....	709.650	50.338	659.312	276.872	382.440	58.01
Crude fat.	235.500	17.370	218.130	80.125	138.005	63.27
Crude fiber.....	1647.000	34.101	1612.899	668.824	944.075	58.53
Pentosan.....	1141.725	56.009	1085.716	500.864	584.852	53.87
N. free extract.	3472.875	349.875	3123.000	771.008	2351.992	75.31
Starch.....	2310.675	238.203	2072.472	189.595	1882.877	90.85
Glucose.	282.600	6.650	275.950	11.734	264.216	95.75
Crude ash.	360.600	22.408	338.192	249.314	88.878	26.28
Nutritive ratio	—	—	—	—	—	9.49

TABLE V. Digestion-coefficient.

	Period I.	Period II.	Increase or decrease in Period II.
Dry matter	64.44%	65.62%	+1.78
Crude protein.....	56.51	58.01	+1.50
Crude fat.....	61.13	63.27	+2.14
Crude fiber	57.95	58.53	+0.58
Pentosan	59.02	53.87	-5.15
N. free extract.....	74.24	75.21	+0.97
Starch.	89.92	90.85	+0.83
Glucose.....	95.02	95.75	+0.63
Crude ash.....	24.83	26.28	+1.45

We may conclude from these data that the addition of takadiastase did not increase the digestibility of starch. As the nutritive ratio has become narrower, being 9.57 in Period I and 9.49 in Period II, it is evident that the digestion of crude fiber was comparatively more accelerated than that of starch. To know whether the secretion of digestive enzymes in the animal body decreased by the addition of digestive enzyme to fodder, protein soluble in pepsin-HCl was estimated according to Pfeiffer's method, and was found as follows :—

	Period I.	Period I.
Total protein.....	12.388%	12.388%
Indigestible protein.....	10.296	10.296
Digestible „	2.092	2.092

The quantity of digestible protein was quite the same in the two periods, from which we infer that the addition of takadiastase did not decrease the secretion of digestive enzymes in the animal body. But as I thought that the negative result obtained in the previous experiment might be due to the too small dose of the enzyme, I repeated the experiment taking a larger doze than in the former experiment. In November 1911 the experiment was repeated with two castrated Merino rams. The fodder given was the same as in the former experiment; viz—hay of wild cereal grass, wheat bran and batatas starch. At first 1kg. of fodder was given to the sheep for a day, but as they ate well, it was increased to 1.2kg, and 2–3 litres of water were given. After the preparatory feeding of 7 days, the dung bags and urine funnels were fastened to the sheep. Sheep A was in sound health and his live weight increased, but sheep B seemed to have somewhat disturbed digestive organs, as the dung excreted was abnormally soft. Many starch granules were observed in the dung of both sheep when stained with iodine. The experiment extended to 20 days. The live weight of the sheep and the quantity of fodder taken, water drunk, feces and urine were as follows :—

TABLE VI.

Sheep A.

Date.	Nov. 25	26	27	28	29	30	Dec. 1.	2	3	4	Total.
Live weight (kg.)..	35.4	35.0	35.0	35.0	35.9	36.3	36.6	37.0	37.9	38.2	
Fodder (gr.).....	999.0	999.0	999.7	1198.0	1199.8	1199.5	1198.0	1198.0	1188.0	1192.0	11371.0

Water (c.c.).	1760	1945	1875	1975	2973	2690	2820	2435	2985	2375	23833.0
Feces (gr.).....	901	823	935	778	909	848	1075	931	747	1025	8972
Urine (c.c.).	 570	565	940	980	780	625	1005	980	1230	825	8440

Sheep B.

Date.	Nov.	25	26	27	28	29	30	Dec.	1	2	3	4	Total.
Live weight (kg.).		38.8	28.5	38.9	38.5	39.1	40.4		40.8	40.9	40.7	41.5	
Fodder (gr.)		998.0	994.0	993.0	1194.0	1188.0	1187.0	1192.0	1192.0	1190.	1182.0	11310.0	
Water (c.c.)		1960	1225	1980	1900	2980	2975	2665	1855	1920	2465	21425	
Feces (gr.)		9.64	805	840	754	1001	971	1075	952	1177	961	9600	
Urine (c.c.)		380	400	390	335	275	375	765	575	455	560	4510	

At the end of the main feeding the bags and urine funnels were removed, and 1gr. of takadiastase was added to the fodder, 0.5 gr. of the enzyme each morning and evening. The takadiastase used was manufactured by Sankyo Co. and had a strong saccharifying power. It contained 29.97% of carbohydrate calculated as starch. After the preparatory feeding of 7 days the dung bags and urine funnels were fastened to the sheep. Both sheep seemed to have better appetite than before, but the fodder was not increased above 1.2 kg. Starch granules were easily observed in the dung. The live weight of the sheep, and the quantity of fodder taken, water drunk, feces and urine as follows:—

TABLE VII.

Sheep A.

Date.	Dec.	11.	12	13	14	15	16	17	18	19	20	Total.
Live weight. (kg.).		37.2	37.5	37.5	37.5	37.5	37.5	37.5	38.0	39.0	39.8	
Fodder (gr.)		1197.0	1200.0	1200.0	1200.0	1200.0	1200.0	1200.0	1200.0	1200.0	1187.0	11984.0
Water (c.c.)		2615	2890	2665	2780	2935	2780	1885	2325	2340	2205	2442.0
Feces (gr.)		1110	1185	1321	1307	1208	1173	1018	728	715	862	10627
Urine (c.c.)		1700	1050	700	710	585	1315	460	825	1305	1055	9705

Sheep B.

Date.	Dec.	11.	12	13	14	15	16	17	18	19	20	Total.
Live weight. (kg.).		41.0	41.5	41.5	41.5	41.5	41.5	41.5	41.2	41.5	42.0	
Fodder (gr.)		1194.0	1200.0	1200.0	1200.0	1200.0	1200.0	1200.0	1200.0	1200.0	1199.0	11993.0
Water (c.c.)		1860	2885	2645	2905	2985	2725	1730	2095	1960	2325	24110
Feces (gr.)		1146	808	765	1290	1277	1254	1232	1224	1140	1250	11386
Urine (c.c.)		625	815	1150	625	470	550	675	605	405	425	6345

In this experiment the feces were collected and weighed every morning and $\frac{1}{10}$ of it was dried at a low temperature, and mixed very well at the end of the experiment to serve for analysis. The composition of the fodder was determined by calculation after analysing the three kinds of fodder separately, as it was very difficult to take for analysis a portion containing the three fodders in proper proportion. The sheep ate all the fodder and left only small particles uneaten. The methods of analysis were the same as in the former experiment. Starch was determined at first by inverting it by boiling with acid, and from the glucose formed being determined by PAVY's method the starch was calculated. The composition of the fodder and feces was as follows:—

TABLE VIII.

FODDER.

	Hay.	Starch and wheat bran	The mixture.
Water.....	5.790%	11.070%	8.880%
Crude protein.....	15.726	10.523	13.125
Protein.....	14.463	9.671	12.067
Crude fat.....	4.537	2.752	3.645
Crude fiber.....	29.930	4.295	17.113
N. free extract.....	36.567	67.900	52.234
Crude ash.....	7.450	2.560	5.005
Starch.....	14.584	63.379	38.982
Organic matter.....	—	—	86.115

FECES.

	Sheep A.		Sheep B.	
	Period I.	Period II.	Period I.	Period II.
Water.....	7.265%	7.765%	6.805%	5.320%
Organic matter.....	80.772	81.365	80.070	82.183
Crude protein.....	15.516	15.446	14.954	15.673
Protein.....	14.112	14.197	13.332	13.266
Crude fat.....	5.740	5.905	4.125	4.855
Crude fiber.....	23.135	22.980	24.225	23.370
N. free extract.....	26.379	37.034	36.766	38.285
Crude ash.....	11.963	10.870	13.125	12.497
Starch.....	13.937	10.434	13.332	11.649

The quantity of air-dry feces was as follows :—

	Period I.	Period II.
Sheep A.....	3692.0 gr.	3895.0 gr.
Sheep B.....	3911.5	3771.5

The digested nutrients and digestion-coefficient were calculated as follows :—

TABLE IX.

PERIOD I.

Sheep A.

	In fodder.	In feces.	Digested.
Organic matter	9792.14 gr.	2982.10 gr.	6810.04 gr.
Crude protein.....	1472.44	572.85	519.59
Protein.....	1372.14	521.02	851.12
Crude fat.....	414.47	211.90	202.57
Crude fiber.....	1945.92	854.14	1091.78
N. free extract.....	5939.53	1343.11	4596.42
Starch.....	4432.64	514.55	3918.09
Crude ash.....	596.12	441.67	154.45
Nutritive ratio.....	—	—	6.72

Sheep B.

	In fodder.	In feces.	Digested.
Organic matter.....	9739.67 gr.	3131.94 gr.	6607.67 gr.
Crude protein.....	1484.44	594.93	889.51
Protein.....	1371.34	521.48	849.86
Crude fat.....	412.25	161.35	250.90
Crude fiber.....	1935.48	947.56	987.92
N. free extract.....	5907.67	1438.10	4469.57
Starch.....	4408.86	521.48	3887.38
Crude ash.....	566.07	513.38	52.69
Nutritive ratio.....	—	—	6.82

PERIOD II.

Sheep A.

	In fodder.	In feces.	Digested.
Organic matter.....	10320.02 gr.	3169.17 gr.	7150.85 gr.
Crude protein.....	1572.90	601.62	971.29

Protein.....	1446.11	552.87	893.23
Crude fat.....	436.82	230.00	206.82
Crude fiber	2050.82	89.51	1155.75
N. free extract.....	6259.72	1442.77	4816.95
Starch	4671.61	406.46	4265.20
Crude ash.	599.80	423.39	176.41
Nutritive ratio.	—	—	6.57

Sheep B.

	In fodder.	In feces.	Digested.
Organic matter.....	10327.78 gr.	3099.53 gr.	7228.24
Crude protein.....	1574.08	5911.07	982.97
Protein.	1447.20	5003.27	946.87
Crude fat.....	437.14	193.11	244.04
Crude fiber.....	2052.36	881.40	1170.96
N. free extract.	6264.42	1443.92	4820.50
Starch.....	4675.11	439.34	4235.77
Crude ash.	600.25	471.32	128.93
Nutritive ratio.	—	—	6.70

DIGESTION-COEFFICIENT.

	Period I.		Period II.	
	Sheep A.	Sheep B.	Sheep A.	Sheep B.
Organic mattar.....	69.54%	67.84%	69.29%	69.99%
Crude protein.....	61.62	59.92	61.75	62.45
Crude fat.....	48.87	60.86	47.34	55.83
Crude fiber.....	56.11	51.04	56.36	57.04
N. free extract.	77.37	75.66	75.66	76.95
Starch	88.39	88.17	91.30	90.60
Crude ash.	21.36	9.31	29.41	21.48

We see from these data that there was some difference in the digestion-coefficient of crude fat, crude fiber, and crude ash between the two experimental animals, but that of the other nutrients agrees well in the two animals. The increase and decrease of the digestion-coefficient in Period II are shown in the following figures :—

TABLE X.

	Sheep A.	Sheep B.
Organic matter.....	-0.25%	+ 2.53%
Crude protein.	+0.13	+ 1.85
Crude fat.....	-1.53	- 5.03
Crude fiber.....	+0.25	+ 6.00
N. free extract.	-0.41	+ 1.30
Starch.....	+2.91	+ 2.43
Crude ash.	+8.05	+12.17

It is seen from these data that the digestion of starch was increased in Period II in both animals ; and as the digestion of crude fiber increased too, it is clear that the increase of the digestion of starch was not caused by the decrease of the digestion of crude fiber. But the nutritive ratio was for the sheep A 6.72 in Period I and 6.57 in Period II, and for the sheep B 6.82 in Period I and 6.70 in Period II. As the nutritive ratio became narrower in Period II it may be assumed that the digestion of protein was comparatively more accelerated than that of starch. The nitrogen equilibrium during the experiment is shown in the following table :—

	Sheep A.		Sheep B.	
	Period I.	Period II.	Period I.	Period II.
N. in urine.	0.9016%	0.8232%	2.1056%	1.6016%
	76.095 gr.	79.892 gr.	94.963 gr.	101.522 gr.
N. resorbed.....	147.134 gr.	155.405 gr.	143.922 gr.	157.275 gr.
Gain by the animals.	71.039 gr.	75.613 gr.	48.959 gr.	55.753 gr.

Nitrogen was deposited in the animal body both in Period I and Period II. Hence we know that the increase of body weight is due not only to the increase of fat, but also to that of protein.

The calorie of the fodder and feces was estimated by Berthelots' bomb-calorimeter, and the calorie resorbed in the animal body was calculated as follows :—

In 1 gr. of dry matter of	
Fodder.....	3359.4 cal.
Feces	
Sheep A {	Period I 4177.5
{	,, II.....4117.2
Sheep B {	Period I.....4135.0
{	,, II.....4241.6

	Sheep A.		Sheep B.	
	Period I.	Period II.	Period I.	Period II.
Fodder.....	43885.2 Cal.	46150.8 Cal.	43649.8 Cal.	46285.8 Cal.
Feces.....	15423.3	16036.5	16174.1	15997.2
Difference.....	28461.9	30114.3	27475.7	30268.6
„ in %.....	64.86	65.25	62.95	65.37
Increase in Period II.	—	0.39	—	2.42

To know whether the secretion of digestive enzymes is decreased by the intake of takadiastase, the protein soluble in pepsin-HCl was determined, and the result was as follows :—

	Sheep A.		Sheep B.	
	Period I.	Period II.	Period I.	Period II.
Total protein.....	16.731%	16.746%	16.046%	16.533%
Soluble protein	0.215	0.216	0.239	0.230
In % of total protein.....	1.285	1.231	1.487	1.390

As soluble protein decreased a little in Period II, it may be assumed that takadiastase diminished sparingly the secretion of digestive enzymes. The results of these experiments show that the addition of a very small dose of a digestive enzyme to the fodder of a healthy animal has no effect on the digestion, but when takadiastase is given in a quantity corresponding to the forty thousands part of the live weight, the digestion of starch is a little accelerated by it, and that of protein is also increased. However, as the increase of the digestibility of starch is very little, it is not economical to use any digestive enzyme in the feeding of domestic animals. The effect of amylatic enzyme such as takadiastase on the fermentation in the digestive tracts was not studied in these trials, which question remains necessarily to be solved later on. The author expresses his hearty thanks to Messrs R. TAKAHASHI and T. INOUE, Assistants of the Agricultural College, who assisted him in these experiments.

The Change of Amino-Acids and Other Constituents of "Koji"-Extract by *Willia anomala* var. saké I, II, III, IV¹, during Fermentation.

BY

Teizo Takahashi.

The occurrence of some varieties of *Willia anomala* as aging yeast of "saké" was reported in a former number of this journal². Their special assimilability of amino-acids and several fermentation products are mentioned in the same report, however, the change of these fermentation products in the course of time affords us some very interesting suggestions as regards the foreseeing of the "saké" disease known as "Hyochi."

As pabulum 300 cc. of the sterilized "koji-extract" (13°B) was used and after infecting four varieties separately it was held at 25–27°C. during 45 days, and the analyses were made four times with an interval of a week or fortnight.

The results are tabulated below :

I. Analysis after 10 days.

	Alcohol.	Total acids.	Amino-acids.	Esters.
	%	%	%	%
Original sol.	—	0.059	0.169	0.470
Culture of Variety I.....	2.72	0.179	0.064	0.308
„ II.....	3.42	0.197	0.082	0.382
„ III.....	2.37	0.268	0.101	0.279
„ IV.....	3.42	0.159	0.071	0.385

II. Analysis after 17 days from the beginning.

Culture of Variety I.....	2.02	0.229	0.098	0.672
„ II.....	2.72	0.189	0.098	0.532
„ III.....	2.02	0.286	0.112	0.473
„ IV.....	3.07	0.64	0.116	0.476

1. In the former report (Journal of the College of Agric. Imp. Univ. Tokyo. Vol. I. No. 3), they are described simply as *Willia anomala* var. I, II, III, IV.

2. Journal of the College of Agric. Imp. Univ. Tokyo. Vol. I. No. 3.
[Jour. Coll. Agric., Vol. V, No. 3, 1915.]

III. Analysis after 31 days from the beginning.

	Total acids.	Amino-acids.	Esters.
Culture of Variety I.....	0.313	0.117	0.303
II.....	0.262	0.108	0.264
„ III.....	0.255	0.120	0.264
„ IV.	0.283	0.126	0.257

IV. Analysis after 45 days from the beginning.

Culture of Variety I.....	0.241	0.108	0.233
II.....	0.245	0.111	0.233
„ III.....	0.258	0.132	0.236
„ IV.	0.253	0.133	0.226

Thus the amounts of the various fermentation products vary in accordance with the varieties, quite analogous to the former report³. Moreover, the varieties I and IV, which give a rather shorter aging period than other varieties assimilate amino-acids more quickly, while the increase of the acidity is not so with these varieties. As reported formerly, in the former aging experiment⁴ there was almost no change in the quantity of the total acids. Still further, variety I giving the shortest ageing period among the four varieties and yielding the most palatable beverage in the former aging experiment (duration about one month), gave the least quantities of amino-acids at the last estimation. This fact suggests to us that the quality of “saké” depends, among other things, upon the quantity of amino-acids; the less of these, the better the saké⁵.

The following tables furnish us with a clearer conception of the change of these products with each variety.

No. I. *Willia anomala* var. *saké* I.

	Alcohol. %	Total acids. %	Amino-acids. %	Esters. %
Original sol.....	—	0.059	0.169	0.470
First analysis.	2.72	0.179	0.064*	0.398
Second „	2.02	0.229	0.098	0.672
Third „	—	0.313	0.117	0.303
Fourth „	—	0.241	0.108	0.233

*Coefficient of Assimilation. 62.

3. Journal of the College of Agric. Imp. Univ. Tokyo. Vol. I. No. 3.
4. Do.
5. Journal of the College of Agric. Imp. Univ. Tokyo. Vol. I. No. 3 p. 269.

No. II. *Willia anomala* var. *saké* II.

Original sol.....—	0.059	0.169	0.470
First analysis 3.42	0.197	0.083	0.382
Second „ 2.72	0.189	0.092	0.532
Third „—	0.262	0.108	0.264
Fourth „—	0.245	0.111	0.233

No. III. *Willia anomala* var. *saké* III.

Original sol.....—	0.059	0.169	0.470
First analysis 2.37	0.268	0.101(coef. of ass. 40)	0.325
Second „ 2.02	0.286	0.112	0.279
Third „—	0.255	0.120	0.264
Fourth „—	0.238	0.132	0.236

No. IV. *Willia anomala* var. *saké* IV.

Original sol.....—	0.059	0.169	0.470
First analysis 3.42	0.159	0.071	0.384
Second „ 3.07	0.264	0.116	0.476
Third „—	0.283	0.126	0.257
Fourth „—	0.253	0.138	0.223

Thus an increase of amino-acid in the culture is observed after a decrease of certain amounts of it with all varieties, and the second phase of the decrease comes rather sooner in variety I. On the other hand, the decrease of the total acid is rather quicker after the increase of it, though the increase of the acids in the former ageing experiment⁶ of "saké" is not observable. On the degree of the increase of the acids in the fermentation of yeast FERNBACH⁷ and VENTRE⁸ affirmed that the degree is rather less, or a decrease occurs, when we add a certain quantity of acid to the common wort or wine must. Consequently, the presence of an almost constant acidity in the "saké" which was used in the former aging experiment (only ten days aging), is accepted as proven, inasmuch as we observe the amount of total acids to be 0.177 grams and a rather large quantity of alcohol in 100cc. "saké."

6. Journal of the College of Agric. Imp. Univ. Tokyo. Vol. I. No. 3.

7. FERNBACH. Comp. rend. t. 156. p. 97. 1913.

8. Ventre. Comp. rend. t. 157. No. 2. p. 154. 1913.

The quantity of the esters decreases during the first stage of fermentation, after which an increase follows, which corresponds to the stage of the decrease of the alcohol.

Conclusion.

In the alcoholic fermentation of *Willia* yeast, the formation of organic acids prevails at the first stage in accordance with a copious assimilation of the pabulum e.g. amino-acids, but the reverse is the case after a somewhat long duration of the fermentation, i.e. an increase of amino-acids and a decrease of organic acids at the same time. In the observation of the change of amino-acids during the storage of "saké," we frequently find a very sound beverage in spite of some noticeable increase in amino-acids.⁹ Such phenomenon can be very clearly explained, if we assume that the similar change will occur in the aging or ripening of saké.

9. About the foreseeing of the saké disease refer to Jour. Coll. Agric., Tokyo Vol. V. No. 2. p. 111. The "Hyochi"-bacilli:—*Bacillus saprogenes saké*, produce rather large quantities of amino-acids in "saké" during storage.

On the Succinic Acid formed by Saké Yeast.

BY

Seiji Nakamoto.

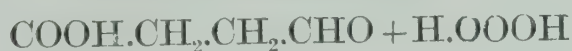
There have been many differences of opinion on the origin of the succinic acid formed in fermentation. It was thought to be formed from the decomposition of sugar by the yeast itself, whilst others traced its formation to the change of asparagin. FELIX EHRLICH has, however, conclusively shown that the succinic acid like fusel oil is in reality derived from amino-acid. The following are the stages in the production of succinic acid from glutamic acid¹:—



Glutamic acid.



Hydroxyglutaric acid.



Succinic semialdehyde. Formic acid.



Succinic acid.

This experimental fact induced me to investigate the yield of the succinic acid from the glutamic acid or its salts, added in the pure state to a solution of sugar, and acted on by different varieties of yeast. So I carried out an experiment with different varieties of saké yeast.

Preparation of the Nutritive Liquids.

The following nutritive media for yeast were prepared.

Nutritive liquid I.

30 grms. Cane-sugar.

1. Über die Entstehung der Bernsteinsäure bei der alkoholischen Gärung. Biochem. Zeitsch. 1909, 18, 391-423.

[Jour. Coll. Agric., Vol. V, No. 3, 1915.]

- 0.3 grms. Monopotassium phosphate.
- 0.9 grms. Magnesium sulphate.
- 0.75 grms. Na-glutamate.
- 300 cc. Distilled water.

Nutritive liquid II (control).

- 30 grms. Cane sugar.
- 0.3 grms. Monopotassium phosphate.
- 0.9 grms. Magnesium sulphate.
- 0.2592 grms. Asparagin.
- 300 cc. Distilled water.

In both media an equal quantity of nitrogen was contained. The asparagin used in this investigation was manufactured by C. MERCK & Co. The sodium glutamate was obtained from "Ajinomoto," manufactured by Suzuki & Co., and was purified by the common method:—changing to hydrochloride of glutamate and afterward into its sodium salt.

At the end of the fermentation, the total and volatile acidity of the liquids was determined. Then the amount of non-volatile acid was calculated from the difference between total and volatile acidity. In this case the non-volatile acid was assumed chiefly as succinic acid produced by yeasts. Of course, all the non-volatile acids formed by yeast are not succinic acid, but as EHRLICH has demonstrated experimentally, it is obvious that the chief part of the acids are succinic acid, when glutamic acid is used as the source of nitrogen.

Methods of experiment :—The pure cultured yeasts were inoculated to the flasks containing the nutritive liquids, kept at room temperature and shaken twice every day. And when the fermentation came to an end, the liquid was filtered and the filtrate used for analysis with the results below :—

Nut. liq.	Variety. of yeast.	Total acid as succinic acid. (%)	Volatile acid as acetic acid. (%)	Non-vol. acid as succinic acid. (%)
I	A 9	0.144	00.27	0.089
II	„	0.095	0.014	0.067
I	A 14	0.159	0.022	0.080
II	„	0.084	0.022	0.041

I	A 21	0.107	0.006	0.094
II	„	0.109	0.021	0.067
I	A 29	0.174	0.027	0.120
II	„	0.138	0.020	0.099
	A 31	0.139	0.023	0.095
II	„	0.114	0.029	0.056
I	B 25	0.136	0.011	0.115
II	„	0.154	0.037	0.080
I	B 30	0.141	0.026	0.089
II	„	0.106	0.014	0.078

Conclusion.

From the preceding results, it is concluded that “saké” yeasts differ in the amount of the production of succinic acid with the variety. The yeasts ranged according to the amount of non-volatile acid produced by them give the following order:—

In the case of solution I.

1.	A 29	0.121
2.	B 25	0.115
3.	A 31	0.095
4.	A 21	0.094
5.	B 30	0.089
6.	A 9	0.089
7.	A 14	0.080

In the case of solution II (control).

1.	A 29	0.099
2.	B 25	0.080
3.	B 30	0.078
4.	A 21	0.067
5.	A 9	0.067

6.	A 31	0.056
7.	A 14	0.041

This experiment was carried out under the direction of Prof. T. TAKAHASHI.

The Fate of Tyrosine in "Shōyu-Moromi."

BY

Matao Yukawa.

One can easily imagine that many complicated chemical changes are going on during the ripening process of "shōyu-moromi." It is evident that these chemical changes are entirely due not only to the various enzymic actions of *Aspergillus Oryzae* in "koji," but also to the vegetative and autolytic actions of many kinds and numbers of microbes in "moromi." Although the chemical and biological compositions have hitherto been isolated from "shōyu" by various authors, the mutual relations between these connected compounds are not yet fully brought to light. Both soy bean and wheat, which serve as the raw materials for "shōyu," are rich in protein matters. When these seed proteins are hydrolysed by means of proteolytic enzymes a tolerable quantity of tyrosine besides a number of amino-acids are always obtained. U. SUZUKI and K. Aso¹, who made elaborate researches on the chemical compositions of "shōyu," presumed the presence of tyrosine in it by PAULY's diazoreaction and MILLON's reaction, the amount being insufficient for identification. On the same paper, however, they stated that tolerable quantities of organic bases occur in "shōyu," and that a new base ($C_6H_9N_3$) and putrescine besides lysine were isolated therefrom. The occurrence of the new base and putrescine in "shōyu" led them to conclude that the two bases are probably the bacterial decomposition products of histidine and arginine respectively, and also that various decomposition products of monoamino-acids by microbes would perhaps be found in it, as the investigation proceeds. According to K. YOSHIMURA² putrescine, ornithine and the formers' new base seem to be the principal bases of "Tamari-shōyu." T. TAKAHASHI³ reported afterwards that trace of tyrosine was left in "miso" analysed by him. I affirmed that this is also the case with "Tamari-shōyu." From these investigations it may be easily conjectured that tyrosine, which was first

1. Bulletin of College of Agric. Tokyo Imp. Univ. Vol. VIII. No. 4.

2. Jour. Coll. Agric. Tokyo Imp. Univ. Vol. I. No. 3.

3. Ibid. Vol. V. No. 2.

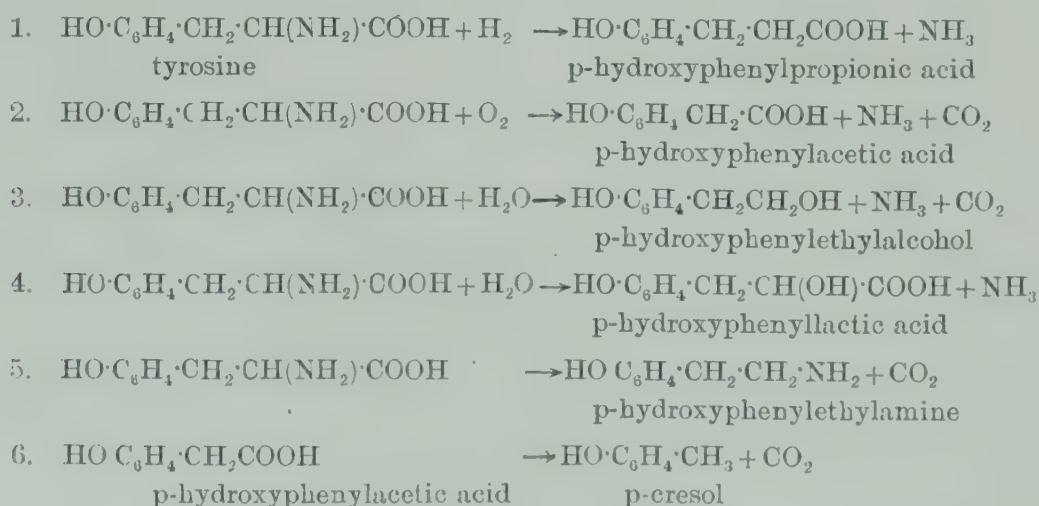
[Jour. Coll. Agric., Vol. V, No. 3, 1915.]

produced from the seed proteins by the proteolytic enzymes of *Aspergillus Oryzae* in the stage of "koji" or in the young stage of the ripening of "moromi," undergoes further decomposition into more simple compounds by the actions of fungi, yeasts or bacteria in "moromi," or most parts of the seed proteins are directly subjected to the actions of the micro-organisms during the ripening process⁴. The shortest way for elucidating this complicated problem is to attack it step by step from the ends of the threads, so I first intended to isolate some decomposition products of tyrosine from "shōyu," and further to throw some light on the formation of the isolated compounds from biological points of view.

Practically, hitherto, p-hydroxyphenylpropionic acid, p-hydroxyphenylacetic acid, p-hydroxyphenylethylamine or tyrosamine, p-cresol and phenol have been identified from the putrefaction products of proteins by several authors. Without question these substances were altogether derived from tyrosine.

Several years ago F. EHRLICH showed that p-hydroxyphenyl-ethylalcohol or tyrosol was formed by yeasts when grown in nutrient solution of tyrosine, and further that p-hydroxyphenyllactic acid was obtained from the same cultured medium in which *Monilia candida* or film yeasts grew.

The decomposition of amino-acids by micro-organisms varies with the different kinds of organisms, the conditions of the growth and the nutrient sources etc. However, the principal decompositions of tyrosine effected by micro-organisms may be expressed by the following reactions :



4. It is also surmised that tyrosine may partially separate out from "shōyu-moromi", being little soluble in water.

Of the decomposition products I have isolated tyrosol and tyrosamine, but not p-hydroxyphenyllactic acid from "shōyu." The latter compound, however, was obtained from the nutrient solution fermented by a *Monilia* species, which was previously isolated from "shōyu-koji" by the author. And further I tried to determine the microbes which play an important rôle on the formation of these compounds.

I. Isolation of some Decomposition Products of Tyrosine.

Tyrosol (p-hydroxyphenylethylalcohol).

Five litres of "shōyu" were evaporated to a small volume at 56°C. under reduced pressure and the separated sodium chloride was removed by filtration. The mother liquor was shaken with a large quantity of 90% alcohol using a shaking apparatus. From the alcoholic extract after filtration, the alcohol was distilled off under a low pressure, and then the most part of the remaining sodium chloride was again removed. The filtrated syrupy liquor was diluted with some water, and after basifying with sodium bicarbonate it was extracted with ether by shaking continuously in a separating funnel.

This operation was repeated until no more was extracted. After evaporating the ether, the extract was again dissolved in a little water and decolorized with animal charcoal, then extracted repeatedly with ether. The extract was evaporated and after priming with a crystal of tyrosol, which was previously obtained from a fermented tyrosine solution, it was allowed to stand for a long time in a vacuum dessicator, then the tyrosol crystallized as bright colourless needles, but the yield was only 0.05 grams.

The crystals have an intensely bitter taste and fragrant odour and are easily soluble in water, alcohol and ether. They give a red colour by MILLON's reagent, and indigo-blue colour by ferric chloride solution. On account of the deficiency of the material only dibenzoate was prepared after SHOTTEN-BAUMER. This compound crystallized as fine needles, which melt at 111°.

From these characteristics it can be concluded that the isolated compound is certainly tyrosol.

The tyrosol contained in "Tamari-shōyu" was by far less than in "shōyu."

p-hydroxyphenyllactic acid.

The basified fluid, from which tyrosol was removed by ether, was made slightly acidic with diluted sulphuric acid, and was continuously shaken with ether as in the case of tyrosol. After evaporation of the ether the residue was dissolved in a little water and decolorised with animal charcoal. The residue gave no crystals, notwithstanding it was dried over concentrated sulphuric acid in vacuum for a long time.

Tyrosamine (p-hydroxyphenylethylamine).

Five litres of "slōyu" were taken for the isolation of this base and the tyrosol was first removed by the preceding method. The basified fluid was added to 200 cc. water, and then gently warmed with five grams of sodium hydroxide until the latter dissolved in it. After cooling it was shaken with a large quantity of ether in a separating funnel continuously. This operation was repeated to extract all the tyrosamine, as it slightly dissolves in ether.

The etheric solution was evaporated, and the syrupy residue diluted with a little water and decolorised with animal charcoal. The aqueous solution was concentrated and dissolved in boiling ether and then saturated with petroleum ether, when colourless needles or platy shaped crystals deposited. The crystals acted strongly basic to litmus and behaved not only to MILLON's reagent and PAULY's reaction, but also distinctly to tryptophol reaction⁵. Therefore it was easily presumed that tryptophol was mingled with these crystals. To remove the former, these were dissolved in hot water and neutralized with diluted hydrochloric acid.

The colourless silky needle-shaped crystals obtained by evaporation were dissolved in methyl alcohol and saturated with ether, and then the separated crystals were filtered. This process was repeated until they became free from tryptophol reaction.

The purified hydrochlorate consists of silky lustered, colourless needles melting at 267°C., soluble in water, alcohol and methyl alcohol, hardly in ether, but not in petroleum ether and ligroin. It becomes red by MILLON's reagent.

5. Reddish violet colour by diaminobenzaldehyde and concentrated hydrochloric acid.

The free base prepared from the hydrochlorate forms bright colourless prismatic crystals which give strong alkaline reaction. It is easily soluble in hot water and alcohol, slightly in cold water and ether. The base becomes strongly red by MILLON'S reagent, also by DENIGE'S test, and gives distinctly PIRIA'S reaction and PAULY'S diazoreaction. It deposits a yellowish precipitate from its solution in bromine water, a voluminous dirty brown precipitate by silver nitrate and baryta, and further precipitates by phosphotungstic acid.

The hydrochlorate was dissolved in a small quantity of boiling water and poured into concentrated chloroplatinic acid solution, and set aside to cool, then needle-shaped or hexagonal plate crystals separated gradually. The chloroplatinate thus obtained was purified by recrystallizing from alcohol.

The chloroplatinate slightly dissolves in alcohol and cold water, but not in ether, and decomposes at 205° in capillary tube.

The analysis of the salt dried in vacuum at 98°C. gave the following results :

0.1167 _g substance	0.032 _g Pt
0.1068 _g „	0.0302 _g Pt
	Pt
	calculated 28.48%
(C ₈ H ₁₁ NOHCl) ₂ PtCl ₄	found 28.10%
	„ 28.27%

The two filtrates from platinum sulphide were put together and evaporating to drive hydrogen sulphide, chlorine was determined by precipitating with silver nitrate solution.

	Chlorine.
found.....	0.0710 _g
„	31.74%
calculated	31.13%

The benzoate prepared from the hydrochlorate is soluble in ether, and alcohol, but not in water or ligroin. It melts at 170°.

The picrate was made from the hydrochlorate. It dissolves in hot water, acetone, ether and alcohol, and melts at 200°C.

According to the above experimental results this base seems to coincide with p-hydroxyphenylethylamine (tyrosamine).

The yield of tyrosamine from "Tamari-shoyu" was 0.1% or so, but from "shōyu" it was about one half of it.

II. Identification of Decomposition Products of Tyrosine
Solution fermented by some Budding Fungi, which were
previously isolated from "Shōyu-Moromi."

As stated in the preceding article I have isolated several kinds of budding fungi from "shōyu-moromi" of different ripening stages. F. EHRLICH has already given a solution upon the decomposition of tyrosine performed by some kinds of yeast and fungus; however, it may be of use to ascertain the influence of my yeasts on the fermentation of tyrosine.

So I prepared three different nutrient media which were composed of

	A	B	C
Water.	100	100	100
Sugar.....	2	2	—
Alcohol	—	—	2
Tyrosine	0.1	0.1	0.1
Magnesium chloride.....	0.2	0.2	0.2
Sodium chloride	0.1	5.0	0.1
Ferric chloride.....	trace	trace	trace

Each one litre of these solutions was put in a flask of ca. two litres capacity with cotton plugs. After repeated sterilizations each solution was inoculated with a small quantity of each of my yeasts and left at 20–28°C. for three months. Then I tried to isolate the decomposition products from each filtrated solution. The methods for isolation of these compounds were exactly the same as in the case of "shōyu."

The results are described below.

(a) *Zygosaccharomyces soja*.

	A	B	C
Yield of yeast	0.55 _g	0.34 _g	no growth
„ „ tyrosol.....	0.40 _g	0.33 _g	

Tyrosol was recrystallized by adding ligroin to the etheric solution, and its properties agreed with EHRLICH's description. This yeast could not develop in C solution, therefore, this species does not assimilate nitrogen from tyrosine in the nutrient medium containing only alcohol as carbon source.

No trace whatever of p-hydroxyphenyllactic acid and tyrosamine was found in the fermented solutions.

(b) *Zygosaccharomyces major*.

	A	B	C
Yield of yeast.....	0.558 _g	0.30 _g	no growth.
„ „ tyrosol	0.43 _g	0.35 _g	

Neither p-hydroxyphenyllactic acid nor tyrosamine were formed by this yeast.

(c) *Zygosaccharomyces japonicus*.

	A	B	C
Yield of yeast.....	0.33 _g	0.51 _g	no growth.
„ „ tyrosol.....	0.13 _g	0.16 _g	

Tyrosamine was not found in the solution. According to EHRLICH's experiment film yeast seems always to form p-hydroxyphenyllactic acid in tyrosine solution. This compound might also be formed in the culture of my yeast, because the yeast easily forms a characteristic film on any nutrient saccharine solution, and further the yield of tyrosine is markedly inferior compared with that of yeast, judging from the cases of the other two. However, unfortunately the isolation of this compound had to be discontinued owing to an accident.

(d) *Monilia* sp.

In contrast to the above three *Zygosaccharomyces* species this fungus developed vigorously in the three nutrient media, and further produced p-hydroxyphenyllactic acid with tyrosol.

	A	C
Yield of fungus.	2.9 _g	1.45 _g
„ „ tyrosol.	0.04 _g	0.09 _g
„ „ p-hydroxyphenyllactic acid...	0.45 _g	0.4

The p-hydroxyphenyllactic acid thus obtained consists of beautiful prisms melting at 169°C. This acid has a characteristic acidic taste and becomes red by MILLON's reagent, bluish yellow by ferric chloride solution and yellow by UFFELMANN's reagent. Tyrosamine was also absent in the fermented solution.

(e) *Mycoderma* sp.

	A	C
Yield of yeast	2.6 _g	3 _g
„ „ tyrosol	0.8 _g	0.5 _g
p-hydroxyphenyllactic acid ...	present	present

This yeast did not form tyrosamine from tyrosine.

Summary.

1. Both tyrosol and tyrosamine were isolated from "shōyu" and "Tamari-shōyu," whilst p-hydroxyphenyllactic acid was never found in them.

2. "Shōyu" contains more tyrosol and less tyrosamine compared with "Tamari-shōyu."

3. The occurrence of tyrosol in "shōyu" or "Tamari-shōyu" is partly or entirely due to the decomposition of tyrosine by the budding fungi dwelling in "moromi."

4. The experimental results, according to which the cultured budding fungi have never produced tyrosamine from tyrosine, led me to presume that tyrosamine in "shōyu" or "Tamari" is certainly produced from tyrosine or its decomposed products formed by *Aspergillus Oryzae* or directly from protein matters by the actions of the same bacteria in "moromi."

5. The absence of p-hydroxyphenyllactic in "shōyu" and "Tamari-shōyu" coincides with the fact that *Monilia* and *Mycoderma* species, which can produce this compound from tyrosine, were isolated only in "koji."

6. In our country "shōyu" or "Tamari-shōyu" are used in cookery as a necessary seasoning on account of their content of amino-acids, carbohydrates, sodium chloride, etc. Moreover, they seem to act as stimulants. The occurrence of tyrosamine in "shōyu" and "Tamari shōyu" makes me easily recognise

this. According to the descriptions of BARGER⁶, DALE and DIXON⁷, and BICKEL and PAWLOW⁸ p-hydroxyphenylethylamine (tyrosamine) causes a contraction of the blood vessels and a rising of blood pressure. Judging from the pharmacological significance of this compound, the consumer of "shōyu" or "Tamari-shōyu" receives a moderate stimulation on his blood circulation.

6. Jour. Chem. Soc. London 95. 1123-28.

7. Jour. of Phys. 39. 25-44.

8. Biochem. Zeitschr. 47. 345-354.

On the Detection of Methylalcohol in Alcoholic Beverages.

BY

Teizo Takahashi.

Of the occurrence of methylalcohol in alcoholic beverages, instances have been reported from time to time. In 1895 WINDISCH¹ detected it in a cherry-brandy, TRILLAT² mentions that he found it in the spirit made from the residue of the grape and other fruits, WOLF³ determined it in several kinds of fruit wine after TRILLAT's and BARDY's methods, moreover, he proceeded to compare the wine prepared from the mash containing the pedicles of the grape and another one which was devoid of them. While in the former 0.15-0.4% of methylalcohol was found, the latter only showed 0.03%. The occurrence of methylalcohol in saké the author has already mentioned in a former bulletin⁴.

The detection or determination of methylalcohol in certain noticeable doses in an alcoholic beverage is very easy, by any method hitherto offered; but when the quantity is very small or if there are only traces of it, the determination gives much trouble.

The official method prescribed by our government and published in 1912 consists of two steps. The first step is DENIGÉ's colorimetric method based on the formation of the violet coloration of the decolorized fuchsin when mixed with a diluted solution of formaldéhyd. But DENIGÉ's process is not applicable with certainty, when methylalcohol is accompanied with other alcohols; for the higher alcohols, such as amylalcohol and others, when oxydised, give very analogous coloration to the decolorized fuchsin and for this reason the second process is necessarily offered to affirm the presence of methylalcohol.

The second step of the method is the urotropin⁵ method applied by E.

1. WINDISCH: Arb. Kais. Ges. Amt. Bd. 11. S. 385, 1895.

2. TRILLAT: Compt. rend. t. cxxviii. p. 438. 440. 1899.

3. WOLF: „ „ „ 131. p. 1323. 1900.

4. Bulletin of the College of Agric. Tokyo. Imp. Univ. Vol. VI. No. 4.

5. Urotropin or hexamethylentetramin. cf. Zeit. anal. Chem. 1896. S. 44-46. P. Dobriner. [Jour. Coll. Agric., Vol. V, No. 3, 1915.]

AWENG⁶ on the proof of methylalcohol in spirit. A small modification of the official method exists in the evaporation of the aldehyd after the addition of ammonia, i.e. hexamethylenetetramin. According to AWENG's direction the evaporation is conducted on the water bath, to which our official method added the condition "below 80°C."

In carrying out the determination the writer noticed that the same sample gave sometimes a positive and in other cases negative results, the only difference being the method of evaporation of hexamethylenetetramin or urotropin. Sometimes very small quantities, such as 1c.c, was added previously to 100 c.c. of the sample, but the proper crystals of urotropin HgCl_2 failed to develop when the temperature of the evaporation was high, and the solution of urotropin colored to brown. Consequently, the condition "*below 80° C*" of the evaporation is not sufficient for the detection of very small quantities or traces of methylalcohol in alcoholic beverages, so in modifying the evaporation "*under reduced pressure, 15-20 mm., and inducing the temperature of the water bath below 45° C*", a constant result was obtained without exceptions. After the modified method methylalcohol was detected in the following samples:—

1. Saké. Trace in many samples.
2. Shōchiu (spirit of sakémash-cake).....Trace, but more than in saké,
3. A Scotch whisky in the market.....More than in Shōchiu.
4. Cognac, Denille Frères, Bordeaux..... " " " "
5. A wine prepared in our laboratory.....Trace.
6. A wine in the market. Trace.

The modified process is as described below.

Take 200c.c of the sample (saké or wine) in a flask of 500c.c. capacity, add 3 gr. of CaCO_3 and distill it with the aid of a fractionating column, below 80°C. The distillation is repeated at as low a temperature as possible (65-70 after AWENG), take 10 c.c, add 250c.c. of 1% K-permanganate and 10 c.c. of sulphuric acid. After 2-3 minutes shaking, decolorize with oxalic acid of 8% and once more distill it, testing from time to time during the process with RIMINI'S or JEAN'S reagents, until the disappearance of the dark-red color formed by the presence of acetaldehyd. The distillate obtained after this test

6. E. AWENG. Apoth. Zeit. 1912. 27 B. S. 159.

7. Still lower temperature reigns in the flask which contains urotropin solution.

is collected as long as it shows a blue color by RIMINI'S reagent. The whole distillate thus obtained is re-distilled after the addition of 3 grams of CaCO_3 . The last distillate is mixed with an excess of ammonia to prepare the urotropin solution, which is *evaporated under reduced pressure (15–20 m.m.) below 45°C.* One drop of the concentrated HgCl_2 solution is taken on the slide, on which one drop of urotropin solution is already at hand. Examine under the microscope.

Über die Bedeutung des Oryzanins für die Ernährung der Gärungsorganismen.

(I. Mitteilung.)

VON

Kanroku Kurono.

Mit Tafel XVI—XVII und 1 Textfigur.

I. Einleitung.

Das Oryzanin ist ein Bestandteil der Reiskleie, welcher Schutz- oder Heilwirkung gegen die durch ausschliessliche Fütterung mit poliertem Reis verursachte Beri-beri ähnliche Krankheit der Tiere hat. Dieser Stoff ist vor einigen Jahre von Prof. SUZUKI in reinem Zustande isoliert und eingehend studiert worden. Er hat die Verbreitung dieses Stoffes in verschiedenen Futtermitteln ermittelt und nach zahlreichen Tierversuchen kam er zu der Annahme, dass das Oryzanin einen unentbehrlichen Bestandteil des Futtermittels bildet. Jedes Futtermittel, dem Oryzanin fehlt, kann das Leben des Tieres nicht längere Zeit erhalten. Auch ein künstliches Futtergemisch aus Eiweiss, Fetten, Kohlenhydrat und Salzen genügt nicht um das Leben des Tieres zu erhalten. Es fehlt noch Oryzanin dazu.¹

Ogleich die chemische Natur dieses höchst interessanten Stoffes und seine physiologische Bedeutung für die höheren Tiere von Prof. SUZUKI und seinen Mitarbeitern immer weiter erforscht und aufgeklärt werden, bleibt eine Seite der Frage noch vollständig unberührt, nämlich die Bedeutung desselben für die Ernährung der niederen Organismen, besonders der Gärungsorganismen. Ich teile hier einige Beobachtungen mit, die ich nach den Ratschlägen des Herrn Prof. SUZUKI in dieser Richtung gemacht habe.

1. U. SUZUKI, T. SHIMAMURA und S. OTAKE:—Biochem. Zeitschrift, Bd. 43, 1912. Ss. 89–153. und dieses Journal, 1913. Vol. I. No. 4. Ss. 382–474.
[Jour. Coll. Agric., Vol. V, No. 3, 1915.]

Als ich mit der Kultur der Hefe mich beschäftigte, habe ich öfters beobachtet, dass Bierhefen, welche vorher in der Würze kultiviert waren, sehr leicht in der Würze wachsen, während sie sich in Koji-extrakt nur langsam vermehren. Gibt man aber 0.01–0.1% Oryzanin zur letzteren Nährlösung zu, so wachsen sie ebenso kräftig wie in der Würze. Verschiedene Kojiarten verhalten sich als Nährböden der Hefe sehr verschieden. In manchen Proben wachsen die Hefen besser als in andern, gibt man aber Oryzanin dazu, so merkt man keinen Unterschied mehr. Diese Tatsache fiel mir auf, und ich glaubte die Ursache derselben dem wechselnden Gehalt an Oryzanin zuschreiben zu müssen. Es ist wohl möglich, dass der Reis, welcher als Material für die Koji-bereitung dient, nicht immer gleichmässig poliert ist, so dass die Menge der anhaftenden Kleie, bezw. des Oryzanins grosse Schwankungen zeigen kann.

Um das Verhalten des Oryzanins auf Hefe genauer kennen zu lernen, ist deshalb unbedingt notwendig, eine absolut oryzaninfreie Nährflüssigkeit zu schaffen. Zu diesem Zwecke eignet sich die künstliche Nährlösung am besten. In der Tat war bei künstlichen Nährböden, wie HAYDUCKscher oder NÄGELscher Lösung, die Wirkung des Oryzanins viel deutlicher zu sehen. Ohne Zusatz von Oryzanin findet in der NÄGELschen Lösung überhaupt keine Vermehrung der Hefe statt. Die HAYDUCKsche Lösung ist etwas besser als die NÄGELsche; trotzdem erweist sich die Kontroll-Lösung gegenüber der oryzaninhaltigen weit ungünstiger. Diese merkwürdige Eigenschaft des Oryzanins lässt an den von WILDIERS als “Bios” bezeichneten Stoff erinnern². Doch behaupten wir gegenwärtig nicht, dass das Oryzanin für die Ernährung der Hefe absolut notwendig ist (wie das Bios Wildiers).

Auf verschiedene andere Heferassen erweist sich das Oryzanin auch ebenso wirksam wie auf Sake- oder Bierhefe.

Die bisher als wachstumreizende Mittel bekannten Substanzen, wie Pepton, Asparagin, verschiedene anorganische Salze u.s.w. stehen in ihrer Wirkung weit hinter dem Oryzanin zurück.

In den meisten Versuchen wurde anstatt reinen Oryzanins der alkoholische Extrakt der Kleie (oder der Hefe) angewendet, so könnte man natürlich fragen,

2. La Cellule, 1901. Bd. 18. S. 313.

ob die günstige Wirkung nicht durch die Verunreinigung des Präparates hervorgerufen sei. Da aber das durch Phosphowolframsäure-Verfahren (nach Prof. SUZUKI) gereinigte Präparat viel wirksamer als der alkoholische Extrakt selbst ist, so kann es nicht der Fall sein.

Nicht nur die Vermehrung der Hefezellen, sondern auch die Bildung des Alkohols wird durch Oryzanin erhöht.

Rohrzucker- oder Rübenzucker-Melasse vergärt sich durch Oryzaninzugabe weit kräftiger, vorausgesetzt, das man etwas Ammonsalze oder Pepton als Stickstoffquelle gleichzeitig zugibt. Oryzanin allein kann keine günstige Wirkung entfalten, weil die Melasse zu arm an Stickstoff ist.

Es ist eine schon längst bekannte Tatsache, dass die Malzkeime, Haspeln, Kleien-Abfälle u.a. in der Brennerei oder Presshefefabrikation öfters der Garflüssigkeit zugegeben werden, um die Gärung zu beschleunigen oder die Produktion des Alkohols zu erhöhen. Die Wirkung dieser sogenannten „indifferenten Stoffe“ sollen nach bisheriger Erklärung nur mechanisch sein, indem sie die Durchlüftung oder die Bewegung der Gärflüssigkeit begünstigen. Wenn man aber aus diesen „indifferenten Stoffen“ durch Extraktion mit heissem Alkohol die oryzaninartigen Stoffe vollständig entfernt, so geht die beschleunigende Wirkung auch beinahe verloren, obgleich die physikalische Beschaffenheit des Rückstandes kaum eine merkbare Veränderung erleidet. Darum nehme ich an, dass die eigentümliche Wirkung nicht mechanisch, sondern der spezifischen Wirkung des Oryzanins zuzuschreiben ist.

Auf verschiedene Bakterienarten wirkt das Oryzanin auch wachstumsreizend, obgleich nicht so deutlich, wie bei Hefearten.

II. Experimenteller Teil.

(I) EINFLUSS DES ORYZANINS AUF DAS WACHSTUM DER HEFE.

Versuch (1). Als Nährflüssigkeit dienten die NÄGELISCHE sowie die HAYDUCKSCHE Lösung. Eine Reihe von kleinen Flaschen von ca. 20 ccm Inhalt (Fig. 3) wurden mit je 10 ccm der betreffenden Nährlösung gefüllt. Einige Flaschen davon wurden mit 0.01% bzw. 0.1% Roh-Oryzanin (alkoholischem Extrakt der Kleie) und einige andere Flaschen mit der entsprechenden

Menge der Asche³ des Rohoryzanins (welche nach dem Veraschen des alkoholischen Extrakts der Kleie zurückbleibt) versetzt, während die übrigen Flaschen als Kontrolle dienten. Jede Flasche wurde mit Watte verschlossen, sterilisiert, und mit Sake, bzw. Bierhefe geimpft. Die Impfung wurde so ausgeführt, dass man eine Platinöse aus dem Bodenabsatz der frisch bereiteten Hefekultur in 10 ccm steril. Wasser verteilt und davon wieder eine Platinnadel voll in jede Flasche überträgt, so dass möglichst wenige Hefezellen in allen Flaschen gleichmässig verteilt werden. Man liess nun die sämtlichen Flaschen in einem Thermostat bei 25–28° stehen. Von Zeit zu Zeit wurden die auftretende Trübung, Schaumbildung oder Absatzbildung u. s. w. sorgfältig beobachtet. Am Ende des Versuches wurden die Zahlen der Hefezellen in 1^{cm} Flüssigkeit mit der THOMASchen Zählkammer ermittelt. Die Ergebnisse sind in den Tabellen (I) und (II) zusammengestellt.

TABELLE I.

HAYDUCKsche Lösung.

Hefearten	Zusatz	Entwicklung nach		Zahl der Hefezellen in 1ccm nach		Bemerkung nach	
		2 Tagen	5 Tagen	2 Tagen	5 Tagen	2 Tagen	5 Tagen
keine Impfung..	0.1% Oryzanin	—	—			klar	klar
Sakehefe..	„	++++		35.8 Mill.	178.0 Mill.	{ Trübung, Absatz, Schaum.	{ Trübung, Absatz
„	„	++++		36.0	164.0	„	„
„	„	++++		25.8	168.0	„	„
„	„	++++		28.0	178.0	„	„
Bierhefe...	„	+++		25.4	128.0	{ wenige Trübung, Abs. S.	„
„	„	+++		26.0	124.0	„	„
„	„	+++		26.0	122.0	„	„
„	„	+++		26.0	122.0	„	„
keine Impfung..	0.01% Oryzanin	—	—			klar	klar
Sakehefe...	„	++		16.4	178.0	{ ziemlich Trübung, Abs.	Absatz
„	„	++		16.0	172.0	„	„
„	„	++		16.8	166.0	„	„
„	„	++		17.0	198.0	„	„

3. Diese Asche bestand hauptsächlich aus Kaliumkarbonat; Spur von Phosphorsäure war auch vorhanden.

Hefearten	Zusatz	Entwicklung nach		Zahl der Hefezellen in 1ccm nach		Bemerkung nach	
		2 Tagen	5 Tagen	2 Tagen	5 Tagen	2 Tagen	5 Tagen
Bierhefe ..	0.01% Oryzanin	++		11.0 Mill.	48.0 Mill.	klar, Absatz.	klar, Absatz
"	"	++		10.8	46.0	"	"
"	"	++		10.6	46.0	"	"
"	"	++		10.8	49.0	"	"
keine Impfung..	Asche des Oryzanins	—	—			klar	klar
Sakehefe..	"	—	+		9.0	"	{ klar, wenig Abs.
"	"	—	+		9.0	"	"
"	"	—	+		8.0	"	"
"	"	±	+		11.0	{ klar, geringer Abs.	"
"	"	±	+		7.0	"	"
Bierhefe ...	"	±	+		1.0	"	"
"	"	±	+		0.9	"	"
"	"	±	+			"	"
"	"	±	+		0.9	"	"
keine Impfung..	ohne Zusatz	—	—			klar	klar
Sakehefe..	"	±	+		12.0	{ klar, geringer Abs.	klar, Absatz
"	"	±	+	0.66	98.0	{ ziemlich trüb, ger. Ab.	"
"	"	±	+		13.0	{ klar, geringer Abs.	"
"	"	±	+		13.0	"	"
Bierhefe ...	"	±	+		7.6	"	"
"	"	±	+		6.0	"	"
"	"	±	+		8.0	"	"
"	"	±	+		6.0	"	"

— bedeutet „keine Entwicklung.“
± „ „spurenweise Entwicklung.“
+ „ „Entwicklung“; die Anzahl der Zeichen gibt die Intensität der Entwicklung an.

TABELLE II.

NÄGELISCHE Lösung.

Hefearten	Zusatz	Entwicklung nach		Zahl der Hefezellen in 1ccm nach		Bemerkung nach	
		2 Tagen	5 Tagen	2 Tagen	5 Tagen	2 Tagen	5 Tagen
keine Impfung	0.1% Oryzanin	—	—			klar	klar
Sakehefe	„	+	++	8.00 Mill.	8.10 Mill.	klar, Absatz	klar, Absatz
„	„	+	++	7.60	8.00	„	„
Bierhefe	„	+	++	2.80	3.00	„	„
„	„	+	++	2.50	2.80	„	„
keine Impfung	0.01% Oryzanin	—	—			klar	klar
Sakehefe	„	+	+	1.60	1.80	klar, Absatz	klar, Absatz
„	„	+	+	1.20	1.30	„	„
Bierhefe	„	+	+	0.95	1.10	„	„
„	„	+	+	0.98	1.14	„	„
keine Impfung	Asche des Oryzanins	—	—			klar	klar
Sakehefe	„	—	—			„	„
„	„	—	—			„	„
Bierhefe	„	—	—			„	„
„	„	—	—			„	„
keine Impfung	ohne Zusatz	—	—			„	„
Sakehefe	„	—	—			„	„
„	„	—	—			„	„
Bierhefe	„	—	—			„	„
„	„	—	—			„	„

Schon zwei Tage nach der Impfung merkte man in allen oryzaninhalten Flüssigkeiten eine ziemlich kräftige Entwicklung, während die Kontrollen noch klar blieben. Nach fünf Tagen war der Unterschied noch deutlicher, manchmal sogar 6–10 mal mehr als in der Kontrolle.

Besonders auffallend war es bei der NÄGELischen Lösung, welche, wenn mit wenigen Hefezellen inokuliert, überhaupt keine Entwicklung zeigte, während durch Zusatz von Oryzavin ein üppiges Gedeihen hervorgerufen wurde.

Der Aschenbestandteil des Rohoryzanins, welcher nach dem Veraschen, allerdings in nur ganz geringen Mengen, zurückbleibt, erwies sich vollständig unwirksam.

Versuch (2). In diesem Versuche wurden die bisher als wachstum-reizende Mittel bekannten Substanzen, wie Eisensulfat, Monokaliumphosphat, Monocalciumphosphat, Ammonphosphat, Ammonsulfat, Magnesiumsulfat, Asparagin, Pepton u. a. mit dem Oryzanin verglichen.

Zu diesem Zwecke wurden je 10 ccm der HAYDUCKSchen Lösung in eine Anzahl von Reagenzgläsern gefüllt, je 0.01% der betreffenden Substanzen zugesetzt und mit Watte verschlossen. Nach Sterilisieren wurden sie mit möglichst wenig Hefezellen geimpft und bei einer Temperatur von 25–28° stehen gelassen.

Das Oryzanin zeigte auch hier die günstige Wirkung ; schon nach zwei Tagen trat in den oryzaninhaltigen Lösungen kräftiges Wachstum ein, während die Kontrolle sowie alle anderen noch vollkommen klar blieben. Nach fünf Tagen wurde der Unterschied noch deutlicher. (Tabelle III.)

TABELLE III.

Nährlösung.	Zahl der Hefezellen in 1ccm nach			
	(Bierhefe)		(Sakehefe)	
	2 Tagen	5 Tagen	2 Tagen	5 Tagen
HAYDUCKSche Lösung ohne Zusatz...	keine Entw.	13.2 Million.	geringe Entw.	10.6 Million.
„ +0.01% Eisensulfat.....	„	13.8	„	10.7
„ +0.01% Magnesiumsulfat.	„	13.5	„	10.6
„ +0.01% Ammonsulfat.....	„	13.0	„	11.5
„ +0.01% Ammonphosphat.	„	13.5	„	10.5
„ +0.01% Monokaliumphos.	„	13.8	„	10.7
„ +0.01% Monocalciumphos.	„	13.0	„	10.5
„ +0.01% Asparagin.....	„	13.2	„	10.6
„ +0.01% Pepton.....	geringe Entw.	14.3	„	11.8
„ +0.01% Roh-Oryzanin.....	11.4	29.2	kräftige Entw.	27.2

Versuch (3). Um festzustellen, ob das Oryzanin auch auf verschiedene andere Heferassen ebenso günstige Wirkung entfaltet, wie bei Sake- oder Bier-

hefe, wurden die folgenden 18 Heferassen durchprobiert, viz: Brennereihefe Rasse II; Brennereihefe, Dänemark; untergärige Bierhefe, Froberg, *Saccharomyces cerevisiae*, Hansen; *Saccharomyces Pasteurianus I*, Hansen; *Saccharomyces Pasteurianus III*, Hansen; *Saccharomyces ellipsoideus I*, Hansen; *Saccharomyces ellipsoideus II*, Hansen; Weinhefe; *Saccharomyces exiguus*, Hansen; *Saccharomyces apiculatus*, Rees; *Saccharomyces Carlsbergensis*; *Zygosaccharomyces major*, Takahashi et Yukawa; *Zygosaccharomyces soja*, Takahashi et Yukawa; *Willia anomala* var. Nachgärungshefe von Sake, Takahashi; *Willia anomala*, Hansen; *Pichia farinosa*; *Pichia rosea*.

Die Versuchsbedingungen waren genau dieselben wie bei Versuch (1) und (2). Je 10 ccm HAYDUCKscher Lösung wurde 0.01% Rohoryzanin (aus Kleie und Hefe) zugesetzt, mit möglichst wenigen Hefezellen inokuliert und bei 25–28° stehen gelassen.

TABELLE IV.

Heiearten	Kleie-Oryzanin		Hefe-Oryzanin		ohne Oryzanin.		
	nach		nach		nach		
	2 Tagen	3 Tagen	2 Tagen	3 Tagen	2 Tagen	3 Tagen	10 Tagen
Brennereihefe, Rasse II.	+++	+++	+++	+++	—	+	
Brennereihefe, Dänemark.....	+++	+++	+++	+++	—	++	
Untergärige Bierhefe.....	+++	+++	+++	+++	—	+	
<i>Saccharomyces cerevisiae</i>	+	++	+	++	—	—	±
<i>Sacch. Pasteurianus I</i>	++	+++	++	+++	—	++	
<i>Sacch. Pasteurianus III</i>	++	++	++	++	+	+	
<i>Sacch. ellipsoideus I</i>	+++	+++	+++	+++	—	++	
<i>Sacch. ellipsoideus II</i>	++	+++	++	+++	—	+	
Weinhefe	+++	+++	+++	+++	—	++	
<i>Sacch. exiguus</i>	++	++	++	++	—	±	±
<i>Sacch. apiculatus</i>	+	++	+	++	—	±	±
<i>Sacch. Carlsbergensis</i>	+	++	+	++	—	—	+
<i>Zygosaccharomyces major</i>	++	++	++	++	—	—	—
<i>Zygosaccharomyces soja</i>	+	++	+	++	—	+	+
Nachgärungshefe von Sake	+++	+++	+++	+++	+++	+++	
<i>Willia anomala</i>	+++	+++	+++	+++	+++	+++	
<i>Pichia farinosa</i>	+++	+++	+++	+++	+++	+++	
<i>Pichia rosea</i>	++	+++	++	+++	++	+++	

Die Ergebnisse waren dieselben wie bei Bier- oder Sakehefe. Eine Ausnahme beobachtete man nur bei Kalmhefen (*Willia-* und *Pichia*-arten), welche in oryzaninhaltiger Lösung kaum besser gedeihen als in der Kontrolle.

Versuch (4). Das in Versuch (1), (2) und (3) angewendete Rohoryzanin-Präparat⁴ wurde weiter gereinigt und auf seine Wirkung geprüft. Zu diesem Zwecke wurden die folgenden 3 Präparate dargestellt.

Präparat (1). Die entfettete Reiskleie wurde in einem Kolben mit dreifacher Menge Alkohol von 85% versetzt und im Rückflusskühler 4 Stunden gekocht. Dann saugte man ab, kochte den Rückstand nochmals zwei Stunden mit gleichen Mengen Alkohol und saugte wieder ab. Die gesamten alkoholischen Auszüge wurden unter vermindertem Druck eingedampft. Derzu rückgebliebene dickbraune Syrup wurde wiederholt mit Aether geschüttelt, um Fette, organische Säuren, Lecithin und andere Verunreinigungen zu entfernen, und weiter bei gelinder Wärme eingedampft. Dieses Präparat⁵ wurde von Prof. SUZUKI als „alkoholischer Extrakt“ bezeichnet.

Präparat (2). Der in oben erwähnter Weise dargestellte „alkoholische Extrakt“ wurde nun in wenig Wasser gelöst und mit Bleiessiglösung gefällt. Das klare Filtrat wurde mit Schwefelwasserstoff behandelt, vom entstandenen Bleisulfid abfiltriert und bei gelinder Wärme eingedampft.

Präparat (3). Der alkoholische Extrakt wurde in wenig Wasser gelöst, mit so viel Schwefelsäure angesäuert, bis sie ungefähr 4% der Flüssigkeit ausmachte und mit 10% Phosphowolframsäure-Lösung versetzt. Der dabei entstandene Niederschlag wurde abgesaugt, mit 4% Schwefelsäure gewaschen, in gewöhnlicher Weise mit Baryt zerlegt und weiter nach dem Verfahren von Prof. SUZUKI verarbeitet. Auf diese Weise bekam man das von ihm als Rohoryzanin⁶ (1) bezeichnete Präparat.

Mit diesen 3 Präparaten wurden folgende Versuche ausgeführt: Je 0.01% der betreffenden Präparate wurde je 10 ccm der HAYDUCKSchen Lösung zugegeben, in gewöhnlicher Weise sterilisiert, und nach der Impfung mit

4. Das Rohoryzanin oder der „alkoholische Extrakt“ enthält nach Prof. SUZUKI eine Anzahl von Substanzen als Verunreinigung, wie z.B. Zucker, organische Säuren, Lecithin, harzartige Substanzen, Kalium, Natrium, nebst einigen, organischen Basen, wie Cholin, Betain, Allantoin, Adenin, Nikotinsäure etc.

5. Die Ausbeute beträgt ca 10% des Ausgangsmaterials.

6. Die Ausbeute des Rohoryzanin (1) beträgt ca 0.4% des Ausgangsmaterials.

wenigen Zellen Sakehefe liess man die sämtlichen Proben bei 25–28°C stehen. Als Kontrolle wurde noch ein blinder Versuch (ohne Hefezusatz) und ein Parallelversuch mit Koji-extrakt ausgeführt (Tabelle V).

TABELLE V.

Nährlösung	Zahl der Hefezellen in 1ccm nach 2 Tagen
Hayducksche Lösung ohne Oryzanin.	4.5 Million
„ „ + 0.01 % Präparat I.	16.6
„ „ + 0.01 % Präparat II.	56.0
„ „ + 0.01 % Präparat III.	78.0
„ „ + 0.02 % Präparat III.	140.0
Kojiextrakt ohne Oryzanin.	72.0

Hier beobachtet man, dass das Präparat (3) am wirksamsten war, dann kommt das Präparat (2); das Präparat (1) war am schwächsten. So ist die Wirkung der Reinheit des Präparates, d. h. dem Oryzaningehalt, direkt proportional.

Versuch (5). Einfluss der Konzentration des Oryzanins auf das Wachstum der Hefe.

Je 10ccm der HAYDUCKSchen Lösung wurden in eine Anzahl Reagenzgläser gefüllt und mit je 0.0001%, 0.001%, 0.01%, 0.03%, 0.06%, 0.1%, 0.25%, 0.5% und 1% Rohoryzanin versetzt, mit Watte verschlossen, sterilisiert und nach der Impfung mit Sakehefe bei 25° aufbewahrt (Tabelle VI).

TABELLE VI.

Nährlösung	Zahl der Hefezellen nach		
	1 Tage	2 Tagen	7 Tagen
Hayducksche Lösung ohne Oryzanin.	—	±	340.0 Million
„ „ + 0.0001 % Oryzanin	—	0.6 Million	600.0
„ „ + 0.001 % „	—	4.3	620.0
„ „ + 0.01 % „	+	25.0	600.0
„ „ + 0.03 % „	++	62.8	640.0
„ „ + 0.06 % „	++	66.2	640.0
„ „ + 0.1 % „	++	54.4	600.0
„ „ + 0.125 % „	++	32.8	650.0
„ „ + 0.5 % „	—	23.6	620.0
„ „ + 1.0 % „	—	10.28	620.0

Wie man sieht, tritt das beste Wachstum bei 0.03–0.06% Oryzanin ein, selbst bei 1% beobachtet man weder verzögernde noch hemmende Wirkung. Die Zahl der inokulierten Hefezellen hat auch grossen Einfluss auf das Wachstum, weil die Hefen selbst nicht unbedeutende Mengen Oryzanin in eigenen Zellen enthalten. Fig. 1 zeigt die Wachstumskurve zwei Tage nach der Impfung. Die Zahl der Hefe als Ordinate und der Prozentgehalt des Oryzanins als Abscisse genommen. Der Maximalpunkt liegt zwischen 0.03 und 0.06%. Wenn man die Kulturflüssigkeiten längere Zeit stehen lässt, so wird der Unterschied immer kleiner und die verschiedenen Proben enthalten schliesslich beinahe die gleiche Anzahl Hefen.

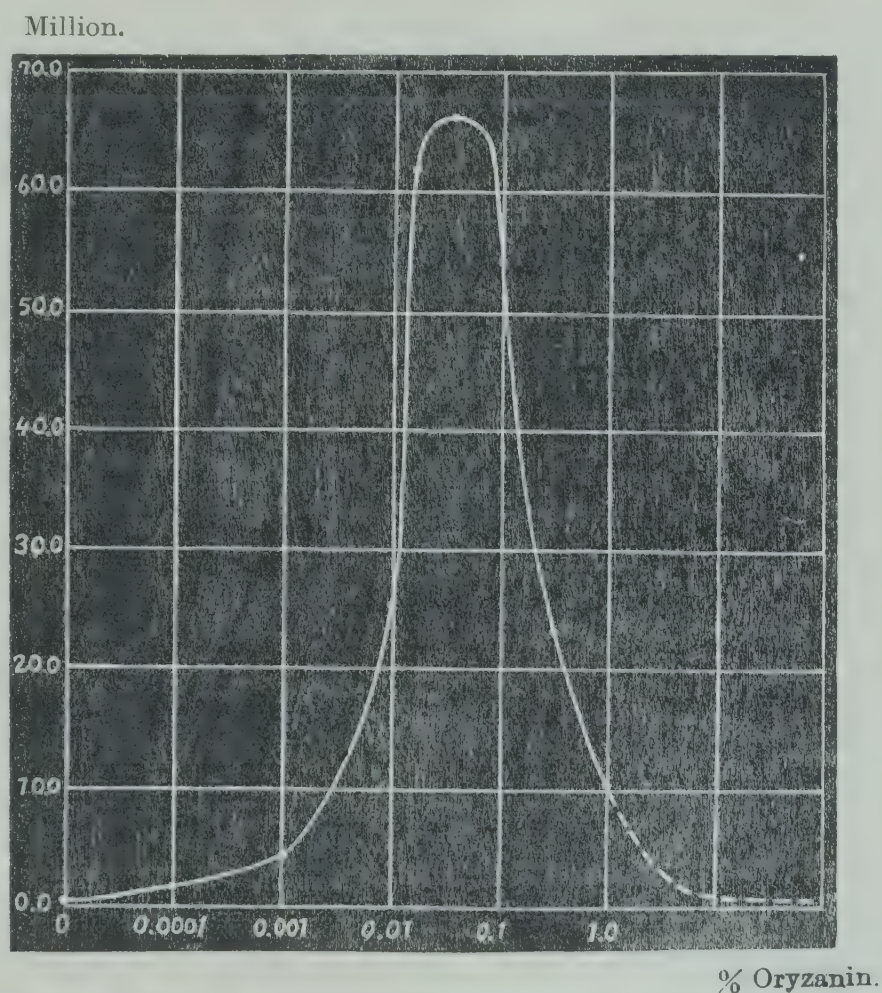


Fig. 1. Sake-Hefe: Wachstumskurve zwei Tage nach der Impfung.

(II) EINFLUSS DES ORYZANINS AUF DIE GÄRKRAFT DER HEFE.

Versuch (6). Gärversuche mit MEISLScher Lösung.

(1). Je 300 ccm MEISLScher Lösung wurden in 4 ERLÉNMEYER-Kolben

von etwa 600 ccm Inhalt gefüllt und mit Chlorcalciumrohr versehen. Zum ersten Kolben gab man 0.1%, zum zweiten Kolben 0.01% Oryzanin zu, während zwei andere als Kontrolle dienten. Jeder Kolben wurde dann mit je 10ccm der frischen Kultur von Sakehefe im Koji-extrakt (12° Bllg.) versetzt und in einem Thermostat bei 25° stehen gelassen. Täglich wurden die Kolben abgewogen, um die Gewichtsabnahme, die durch Entweichen der Kohlensäure verursacht wird, zu ermitteln, und am Ende des Versuches wurde der gebildete Alkohol in den einzelnen Kolben quantitativ bestimmt (Tabelle VII).

TABELLE VII

Gärflüssigkeit	Tägliche Gewichtsabnahme (durch Entweichen von CO ₂) in Gramm.							Alkohol gebildet
	1 Tag	2 Tag	3 Tag	4 Tag	5 Tag	6 Tag	Summe	Vol. %
300ccm MEISLSCHES Lösung ohne Oryz.	2.2	2.35	2.2	1.7	1.2	0.7	10.35	4.2
„ „ „ 0.1% Oryzanin.	3.45	3.2	2.2	1.25	0.6	0.3	11.00	5.5
„ „ „ 0.01% Oryzanin.	3.0	3.0	2.2	1.65	0.7	0.35	10.90	5.0
„ „ „ ohne Oryzanin...	1.9	2.1	2.25	2.0	1.2	0.6	10.05	4.0

Versuch (7). Gärversuch mit Koji-extrakt.

(1) Der gewöhnliche Koji-extrakt (10° Bllg.) wurde mit Rohrzucker versetzt und in 3 Erlenmeyerkolben gleichmässig verteilt. Zum ersten Kolben gab man 0.05% Rohoryzanin zu und zum zweiten 0.05% KH₂PO₄, während der dritte 0.05% (NH₄)₂SO₄ bekam. Nachdem sie mit gleichen Mengen einer frischen Kultur Sakehefe versetzt wurden, liess man sie bei 25–28° stehen. Am Schlusse der Gärung wurden der Alkohol, die Gesamtsäuren und Aminosäuren quantitativ ermittelt.

(2) Derselbe Versuch wurde mit einem Koji-extrakt (12° Bllg.) ohne Zuckerzufuhr wiederholt. Der Oryzaningehalt war 0.1% und die Temperatur 7–15°.

Die Ergebnisse sind in Tabelle VIII zusammengefasst.

Aus diesem Resultat sieht man, dass der Gärprozess auch durch Oryzanin (0.01%–0.1%) etwas beschleunigt wird. Der Alkoholgehalt steigt, obgleich

nur in geringem Grade. Die Gesamtsäuren so wie die Aminosäuren zeigen dagegen eine Abnahme.

TABELLE VIII.

(I)

Gärflüssigkeit.	Tägliche Gewichtsabnahme (durch Entweichen von CO ₂) in Gramm.					Alkohol gebildet	Amino- säuren %	Gsamt- säuren %
	1 Tag	2 Tag	4 Tag	5 Tag	Sum- me	Vol. %	als Gly- cocol	als Milch- säure
300 ccm Kojiextrakt ohne Oryzanin.....	12.8	8.95	2.3	0	24.05	9.0	0.263	0.114
„ „ „ „	11.4	5.0	2.2	0	18.6	8.0	0.285	0.390
„ „ + 0.1% Oryzanin...	14.3	8.0	1.9	0	24.2	10.5	0.220	0.126
„ „ + „ „ ..	14.8	8.0	1.3	0	24.1	9.5	0.240	0.114
„ „ + 0.1% KH ₂ PO ₄ ...	12.8	8.6	2.1	0	23.5	9.0	0.300	0.102
„ „ + 0.1%(NH ₄) ₂ SO ₄ ..	13.0	8.0	2.9	0	23.9	9.0	0.285	0.144

(II)

Gärflüssigkeit	Tägliche Gewichtsabnahme durch Entweichen von CO ₂						Alkohol gebildet
	1 Tag	2 Tag	3 Tag	4 Tag	5 Tag	Summe	Vol. %
300 ccm Kojiextrakt + 0.1% Oryzanin....	1.5	/	/	/	/	/	/
„ „ + „ „	1.6	2.2	3.2	1.8	0.3	9.1	4.0
„ „ ohne Oryzanin	1.3	1.9	2.8	2.3	0.3	8.6	3.5
„ „ „ „	1.4	1.9	2.8	2.3	0.3	8.7	3.5
Zimmertemperatur	9°-13°	10°-12°	7°-14°	10°-15°	10°-14°		

(III) DIE WIRKUNG DER SOGENANTEN “INDIFFERENTEN STOFFE”
AUF DIE MELASSEGÄRUNG.

Es ist eine altbekannte Tatsache, dass die sogenannten “indifferenten Stoffe,” wie Treber, Schalen, Roggenschrot, Malzkeime u. s. w. öfters in der Brennerei und Presshefefabrikation zur Erhöhung der Gärkraft gebraucht

werden. M. OKAZAKI⁷ berichtet auch neulich, dass die Zugabe kleiner Mengen Reiskleie sehr günstigen Einfluss auf die Gärung der Melassemaischen ausübt. Worin liegt aber die Ursache der günstigen Wirkung? DELBRÜCK⁸ schrieb zuerst diese Wirkung dem Eiweissstoffe zu, der als Nährstoff der Hefe dienen soll. Diese Ansicht wurde aber bald von ihm verlassen, indem er die Erklärung in den mechanischen Eigenschaften der indifferenten Stoffe suchen wollte. OKAZAKI stellte auch fest, dass die Fette oder Eiweissstoffe der Reiskleie keine wichtige Rolle spielen. Da die indifferenten Stoffe immer oryzaninreiches Material darbieten, so lässt sich natürlich vermuten, dass das Oryzanin hier auch die Hauptrolle spielt. Die folgenden Versuche zeigen uns, dass es tatsächlich der Fall ist.

Versuch (8). (1) Es wurden folgende 5 Lösungen hergestellt:

1. Melasselösung⁹ allein.
2. „ „ + 0.1% Pepton.
3. „ „ + 0.1% Ammonsulfat.
4. „ „ + 0.1% Ammonsulfat +
0.01% Rohoryzanin (der Kleie).
5. „ „ + 0.1% Ammonsulfat +
0.01% Rohoryzanin (der Hefe).

Je 10 ccm der obigen Lösungen wurden in eine Anzahl Reagenzgläser gefüllt, mit Watte verschlossen, in gewöhnlicher Weise sterilisiert und mit möglichst geringen Mengen der Brennereihefe Rasse II. geimpft. Sämtliche Proben wurden bei 25° stehen gelassen. Tabelle IX zeigt die Beobachtung zwei Tage nach der Impfung.

7. M. OKAZAKI: Journal of Scientific Agric. Society (Japanisch.) 1913, No. 132, No. 135.

8. DELBRÜCK: Woch. f. Brauerei; 1886, S. 306, S. 645; 1887, S. 73. Delbrück u. Hayduck: Die Gärungsführung; 1911, S. 37-42, S. 145-156. Henneberg: Gärungs-Bakteriologisches Praktikum 1909, S. 305-311.

9. Die käufliche Rohrzucker-Melasse wurde mit verdünnter Schwefelsäure neutralisiert, nach kurzem Abkochen klar abfiltriert und mit Wasser auf 22° Bllg. verdünnt.

TABELLE IX.

Nährlösung	Zahl der Hefezellen in 1 ccm	Bemerkung
Melasselösung allein.	5.7 Million.	{ klar, keine Schaumentwicklung, Absatz unbedeutend.
Melasselösung + 0.1% Pepton.	6.0	{ klar, geringe Schaumentwicklung, Absatz unbedeutend.
Melasselösung + 0.1% $(\text{NH}_4)_2\text{SO}_4$	5.9	{ klar, keine Schaumentwicklung, Absatz unbedeutend.
Melasselösung + 0.01% $(\text{NH}_4)_2\text{SO}_4$ + 0.01% Kleie-Oryzanin.	7.0	{ klar, kräftige Schaumentwicklung, viel Absatz.
Melasselösung + 0.01% $(\text{NH}_4)_2\text{SO}_4$ + 0.01% Hefe-Oryzanin.	7.1	{ klar, kräftige Schaumentwicklung, viel Absatz.

Man sieht also, dass die Schaumentwicklung, sowie die Absatzbildung durch Zusatz von Oryzanin + Ammonsulfat (4 und 5) am schnellsten vor sich gehen.

(2) 10 ERLÉNMEYER-Kolben von etwa 600 ccm Inhalt wurden mit je 400 ccm Melasselösung gefüllt und mit 0.2% Ammonsulfat versetzt. Ausserdem wurden noch folgende Substanzen zugegeben, nämlich:—

1. Kontrolle.
2. „
3. 0.2_g Rohoryzanin.
4. 0.05_g „
5. 1.0_g Reiskleie.
6. 2.0_g „
7. 1.0_g Oryzaninfreie Reiskleie¹⁰.
8. 2.0_g „ „
9. 1.0_g Malzkeime.
10. 1.0_g Oryzaninfreie Malzkeime¹¹.

10. Reiskleie wurde dreimal mit 85% Alkohol heiss extrahiert um das Oryzanin zu entfernen.

11. Malzkeime wurden ebenso behandelt wie Reiskleie.

Jeder Kolben wurde mit Chlorcalciumverschluss versehen. Nach dem Sterilisieren wurden sie mit je 1 ccm schleimiger Absatzhefe (Brennereihefe Rasse II) inokuliert und in einem Thermostat bei 25° stehen gelassen. Sie wurden täglich abgewogen, um die Kohlensäureentwicklung zu ermitteln (Tabelle X).

Die Ergebnisse lassen sich kurz wie folgt zusammenfassen: Die Melassegärung wird durch Zusatz von 0.05% Rohoryzanin ziemlich beschleunigt. 0.25% Reiskleie oder Malzkeime wirken ebenso gut wie Rohoryzanin. In diesen Fällen verläuft die Gärung am Anfang sehr kräftig und kommt viel schneller zu Ende als in der Kontrollflüssigkeit.

Wenn man die Reiskleie oder Malzkeime mit heissem Alkohol wiederholt extrahiert, so wird das Oryzanin zum grössten Teil entfernt, und gleichzeitig geht die Reizwirkung beinahe verloren.

TABELLE X.

Gärflüssigkeit.	Tägliche Gewichtsabnahme (durch Entweichen von CO ₂) in Gramm.											
	1. Tag	2. Tag	3. Tag	4. Tag	5. Tag	6. Tag	7. Tag	10. Tag	11. Tag	12. Tag	13. Tag	Summe
400 ccm Melasselösung allein	1.9	3.0	2.2	2.9	2.5	2.0	1.9	3.6	1.1	0.5	0	21.6
„ „ „	1.9	3.1	2.2	2.8	2.4	2.0	1.8	3.7	1.0	0.4	0	21.4
400 ccm Melasselösung + 0.2g Oryzaninextrakt.	9.0	2.8	3.4	3.0	2.4	2.0	1.0	1.5	0.4	0		25.5
400 ccm Melasselösung + 0.05g Oryzaninextrakt.	2.6	3.9	2.6	3.3	3.0	2.3	2.1	2.9	0.4	0		23.1
400 ccm Melasselösung + 4g. Reiskleie.	9.0	12.1	1.0	0.6	0							22.7
400 ccm Melasselösung + 2g Reiskleie.	14.5	7.4	0.5	0								22.4
400 ccm Melasselösung + 1g beinahe oryzaninfreie Reiskleie.	4.9	6.7	5.9	4.3	0.5	0						22.3
400 ccm Melasselösung + 2g beinahe Oryzaninfreie Reiskleie.	5.9	5.6	4.4	4.4	1.4	0.5	0					22.2
400 ccm Melasselösung + 1g Wurzelkeime des Malzes.	6.7	9.2	3.8	2.1	0.6	0						22.4
400 ccm Melasselösung + 1g beinahe oryzaninfreie Wurzelkeime des Malzes.	2.1	4.2	3.7	4.5	4.1	2.5	1.1	0.8	0			23.0

(IV) WIRKUNG DES ORYZANINS AUF BAKTERIEN.

Versuch (9). Um das Verhalten des Oryzanins auf die Ernährung der Bakterien zu studieren wurden die folgenden 4 Nährlösungen hergestellt.

- (1) FRÄNKELSche Lösung,
- (2) „ „ + 0.01% Oryzanin der Kleie,
- (3) „ „ + „ „ der Hefe,
- (4) Nährbouillon.

Je 10 ccm dieser Lösungen wurde in Reagenzgläser gefüllt und in gewöhnlicher Weise sterilisiert. Die folgenden 15 Bakterienarten wurden durchprobiert:—

Proteus vulgaris, Hauser. *Bacterium typhi murium*, Löffler.

Bact. typhi murium, Mereschkowsky. *Bact. denitrificans*, Stutzer u. Burri.

Bact. Pasteurianum, Hansen. *Bact. fluorescence*, Long.

Bacillus mesentericus vulgatus, Flügge. *Bac. coli communis*, Escherich.

Bac. cyanogenes, Flügge. *Bac. subtilis*, Cohn.

Bac. lactis viscosus. *Sarcina albida*, Gruber.

Sarcina aurantica, Flügge u. Lindner. *Micrococcus ureae*, Flügge.

Micrococcus flavus, Flügge.

Diese Bakterienarten wurden mit grosser Vorsicht in die oben erwähnten Lösungen gleichmässig geimpft und bei 25° stehen gelassen. Von Zeit zu Zeit wurden die auftretende Trübung, Absatzbildung oder die Hautbildung u. s. w. sorgfältig beobachtet (Tabelle XI). Aus diesem Versuche sieht man, dass das Wachstum der meisten Bakterienarten mehr oder weniger durch Oryzanin beschleunigt wird. Auf einige, die in der FRÄNKELSchen Lösung sich nicht zu entwickeln vermögen, erwies sich das Oryzanin als unwirksam.

Ferner habe ich 3 Bakterienarten (*Bacillus coli communis*, *B. typhi murium* Löffler und *B. lactis viscosus*) 15 mal nacheinander in reiner FRÄNKELScher Lösung umgezüchtet ohne merkbare Verzögerung oder Verlangsamung des Wachstums zu beobachten. Da die FRÄNKELSche Lösung kein Oryzanin enthält, und die Übertragung des Oryzanins, das eventuell im originellen Nährboden vorhanden sein könnte, durch mehrmaliges Umzüchten vollständig ausgeschlossen ist, so muss man annehmen, dass das Oryzanin

für Bakterienernährung nicht absolut notwendig ist, obgleich es eine Reizwirkung auf dieselben ausübt.¹²

TABELLE XI.

Bakterienarten	Nährboden	Entwicklung nach			
		1 Tage	2 Tagen	3 Tagen	30 Tagen
<i>Proteus vulgaris</i>	Nährbouillon	++	+++	+++	
	Fränkelsche Lösung	—	±	++	
	F. L. + Kleie-Oryzanin	±	++	++	
	F. L. + Hefe-Oryzanin	+	++	++	
<i>Bact. typhi murium</i> , Löffler.	Nährbouillon	++	+++	+++	
	Fränkelsche Lösung	—	—	++	
	F. L. + Kleie-Oryzanin	+	++	++	
	F. L. + Hefe-Oryzanin	+	++	++	
<i>Bact. typhi murium</i> , Mereschkowsky.	Nährbouillon	++	+++	+++	
	Fränkelsche Lösung	—	+	+	
	F. L. + Kleie-Oryzanin	+	++	++	
	F. L. + Hefe-Oryzanin	+	++	++	
<i>Bact. denitrificans</i>	Nährbouillon	+	++	++	
	Fränkelsche Lösung	±	++	++	
	F. L. + Kleie-Oryzanin	+	++	++	
	F. L. + Hefe-Oryzanin	+	++	++	
<i>Bact. Pasteurianum</i>	Nährbouillon	—	±	+	+
	Fränkelsche Lösung	—	—	—	—
	F. L. + Kleie-Oryzanin	—	—	—	—
	F. L. + Hefe-Oryzanin	—	—	—	—
<i>Bact. fluorescence</i> , Long.	Nährbouillon	±	++	+++	
	Fränkelsche Lösung	—	+	++	
	F. L. + Kleie-Oryzanin	—	+	++	
	F. L. + Hefe-Oryzanin	—	+	++	
<i>Bac. mesentericus</i> <i>vulgatus</i>	Nährbouillon	—	+	++	
	Fränkelsche Lösung	—	—	—	—
	F. L. + Kleie-Oryzanin	—	+	+	
	F. L. + Hefe-Oryzanin	—	+	+	
<i>Bac. coli communis</i> ..	Nährbouillon	++	++	+++	
	Fränkelsche Lösung	—	±	+	
	F. L. + Kleie-Oryzanin	±	++	++	
	F. L. + Hefe-Oryzanin	+	++	++	

12. Vergleiche Y. TŌYAMA: “Über die Bedeutung des Oryzanins für die Ernährung der Bakterien.” Zeitschrift für Bakteriologie (Japanisch: Saikingakuzasshi, 1914 August).
Es ist möglich, dass die Bakterien selber oryzaninartige Stoffe aus anderen Substanzen bilden, falls sie solche nötig haben.

<i>Bac. cyanogenus</i>	Nährbouillon Fränkelsche Lösung F. L. + Kleie-Oryzanin F. L. + Hefe-Oryzanin	++ — + +	+++ ++ ++ ++	+++ ++ ++ ++	
<i>Bac. subtilis</i>	Nährbouillon Fränkelsche Lösung F. L. + Kleie-Oryzanin F. L. + Hefe-Oryzanin	— — — —	+ — — +	+ — — +	— — — —
<i>Bac. lactis viscosus</i>	Nährbouillon Fränkelsche Lösung F. L. + Kleie-Oryzanin F. L. + Hefe-Oryzanin	++ — + +	+++ + ++ ++	+++ + ++ ++	
<i>Sarcina albida</i>	Nährbouillon Fränkelsche Lösung F. L. + Kleie-Oryzanin F. L. + Hefe-Oryzanin	— — — —	+ — ± +	+ — ± +	± ± ± ±
<i>Sarcina aurantica</i>	Nährbouillon Fränkelsche Lösung F. L. + Kleie-Oryzanin F. L. + Hefe-Oryzanin	— — — —	+ — — ±	+ — — +	— + — (nach 10 Tagen)
<i>Sarcina ureae</i>	Nährbouillon Fränkelsche Lösung F. L. + Kleie-Oryzanin F. L. + Hefe-Oryzanin	++ — — —	++ — + ++	++ — + ++	— — — —
<i>Micrococcus flavus</i>	Nährbouillon Fränkelsche Lösung F. L. + Kleie-Oryzanin F. L. + Hefe-Oryzanin	— — — —	+ — — +	+ — — +	— + — (nach 10 Tagen)

ERKLÄRUNG DER TAFELN.

TAFEL XVI.

Fig. 2. Sakehefe im Kojiextrakt. Nach zwei Tagen bei 25°C. *A, a*, enthalten 0.01% Hefe-Oryzanin (nach dem Phosphowolframsäure-Verfahren dargestellt). *B, b*, ohne Oryzanin.

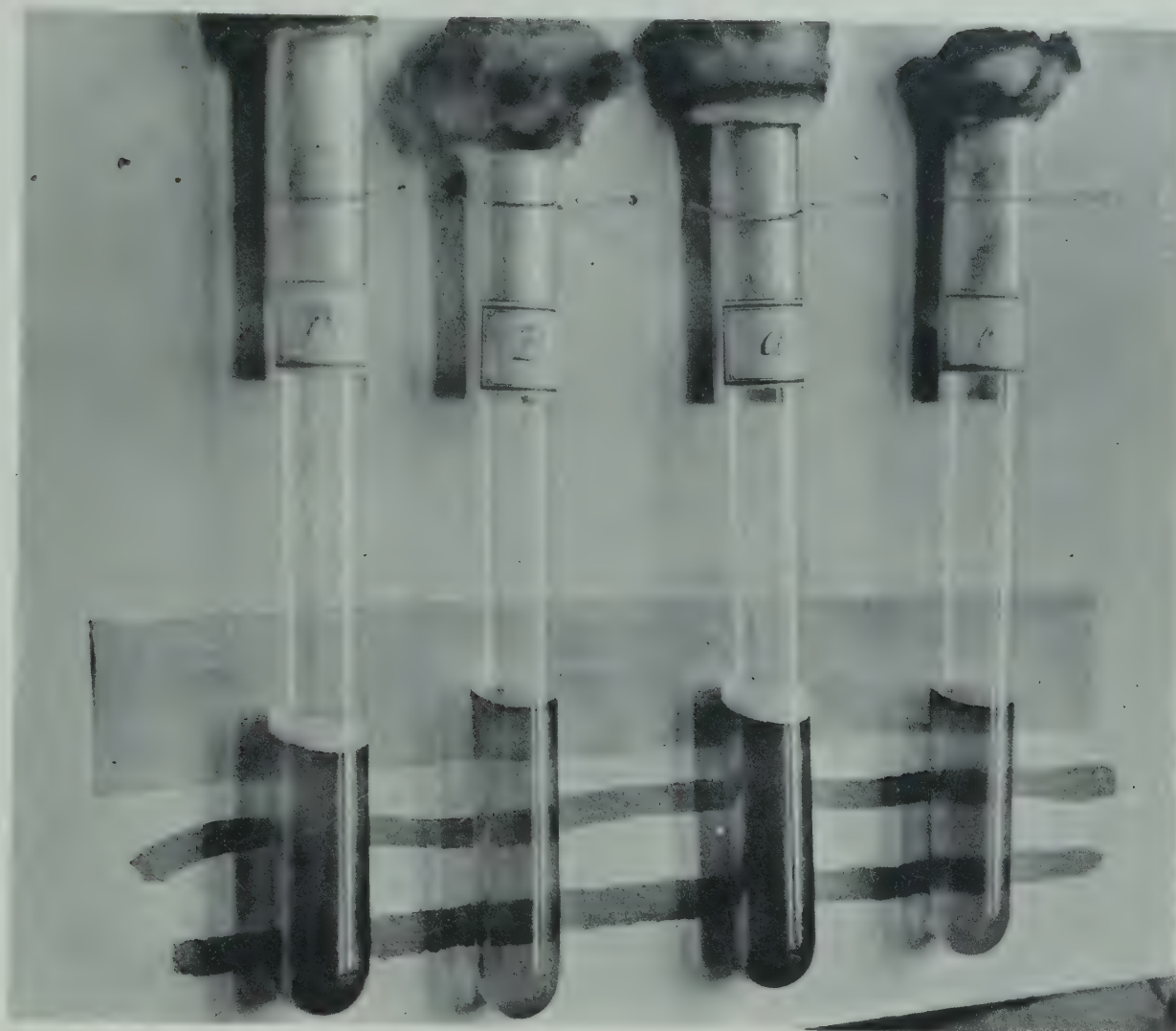
Fig. 3. Sakehefe in Hayduckscher Lösung. Nach zwei Tagen bei 25°C. *A, a*, enthalten 0.1% Kleie-Oryzanin. *B, b*, enthalten 0.01% Kleie-Oryzanin. *C, c*, enthalten entsprechende Menge der Asche des Kleie-Oryzanins. *D, d*, Hayducksche Lösung allein.

TAFEL XVII.

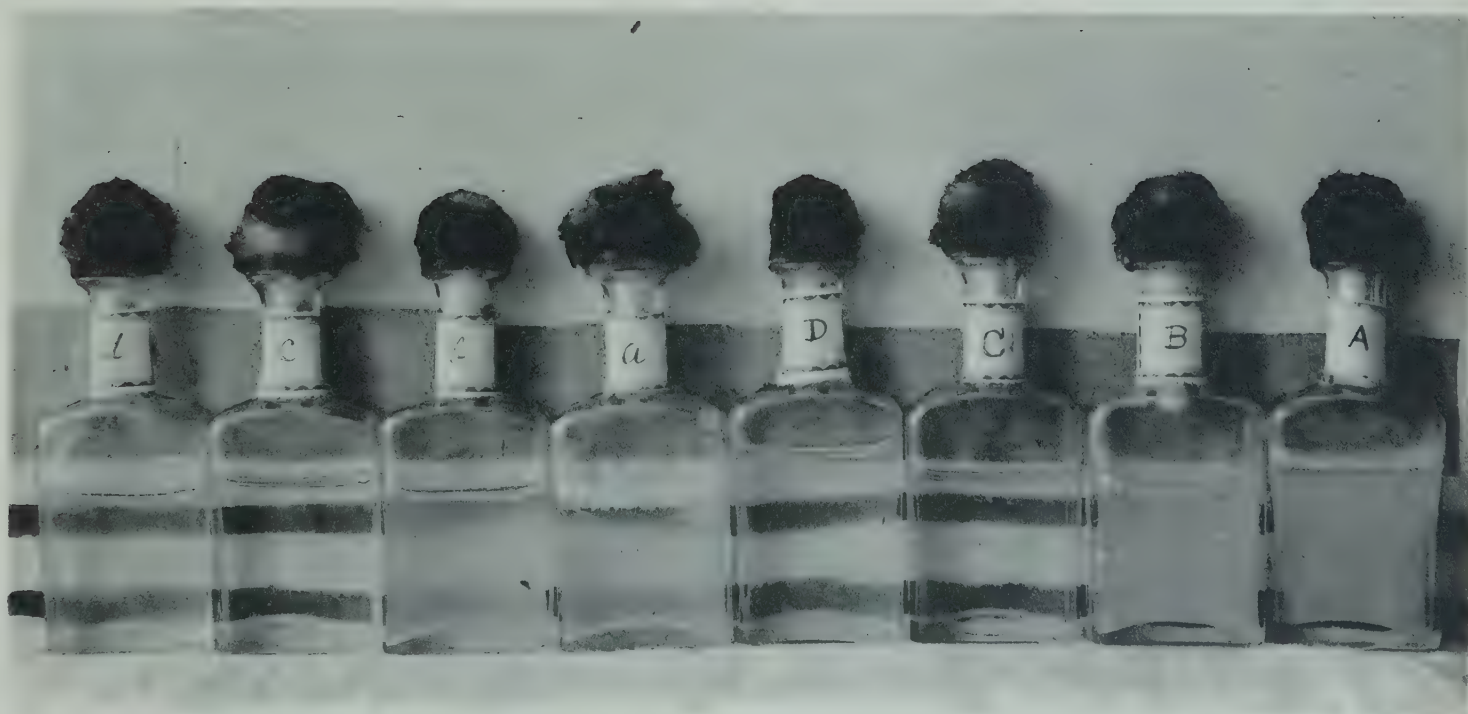
Fig. 4. Weinhefe (1, 2, 3) und *Saccharomyces ellipsoideus*. I. (4, 5, 6) in HAYDUCKScher

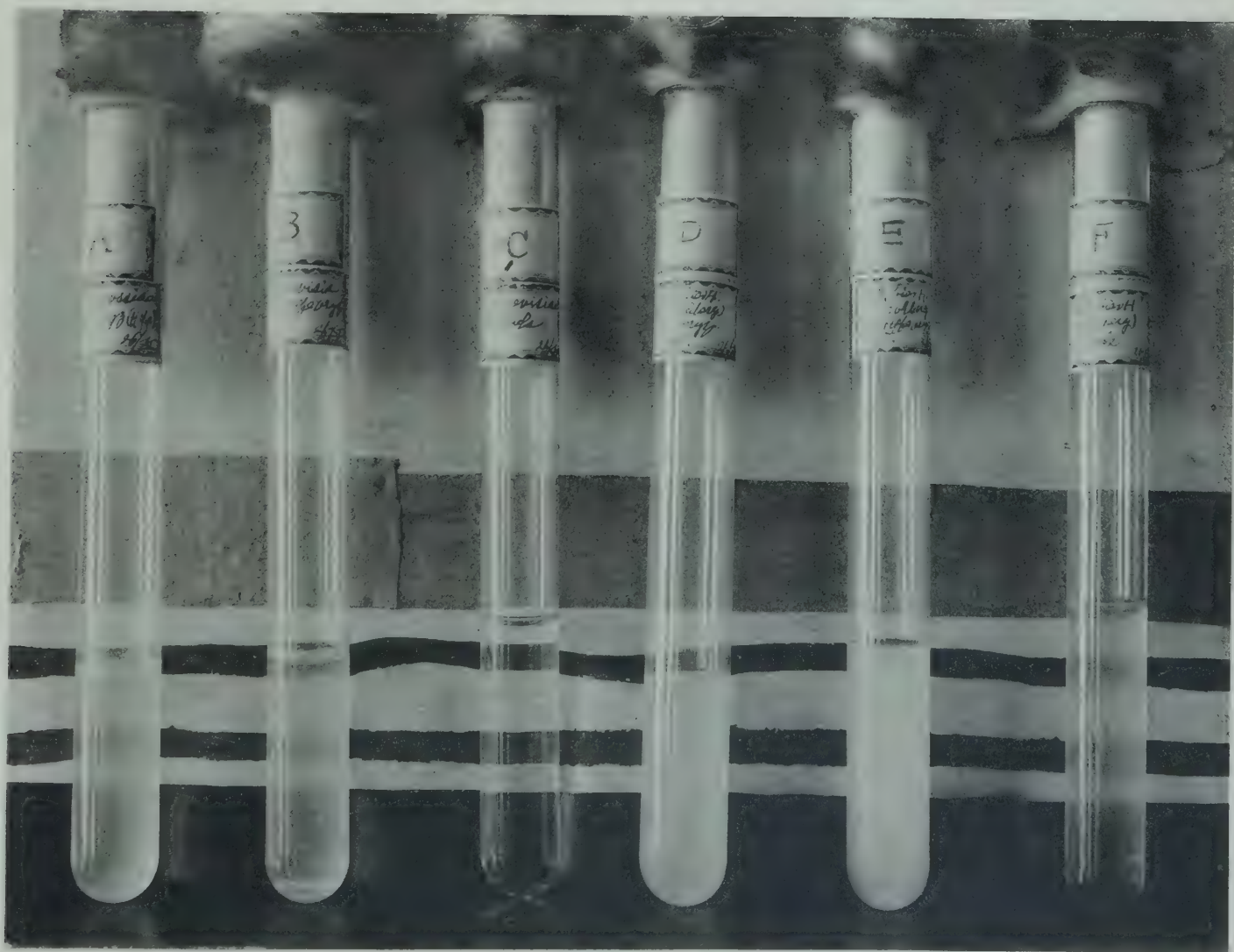
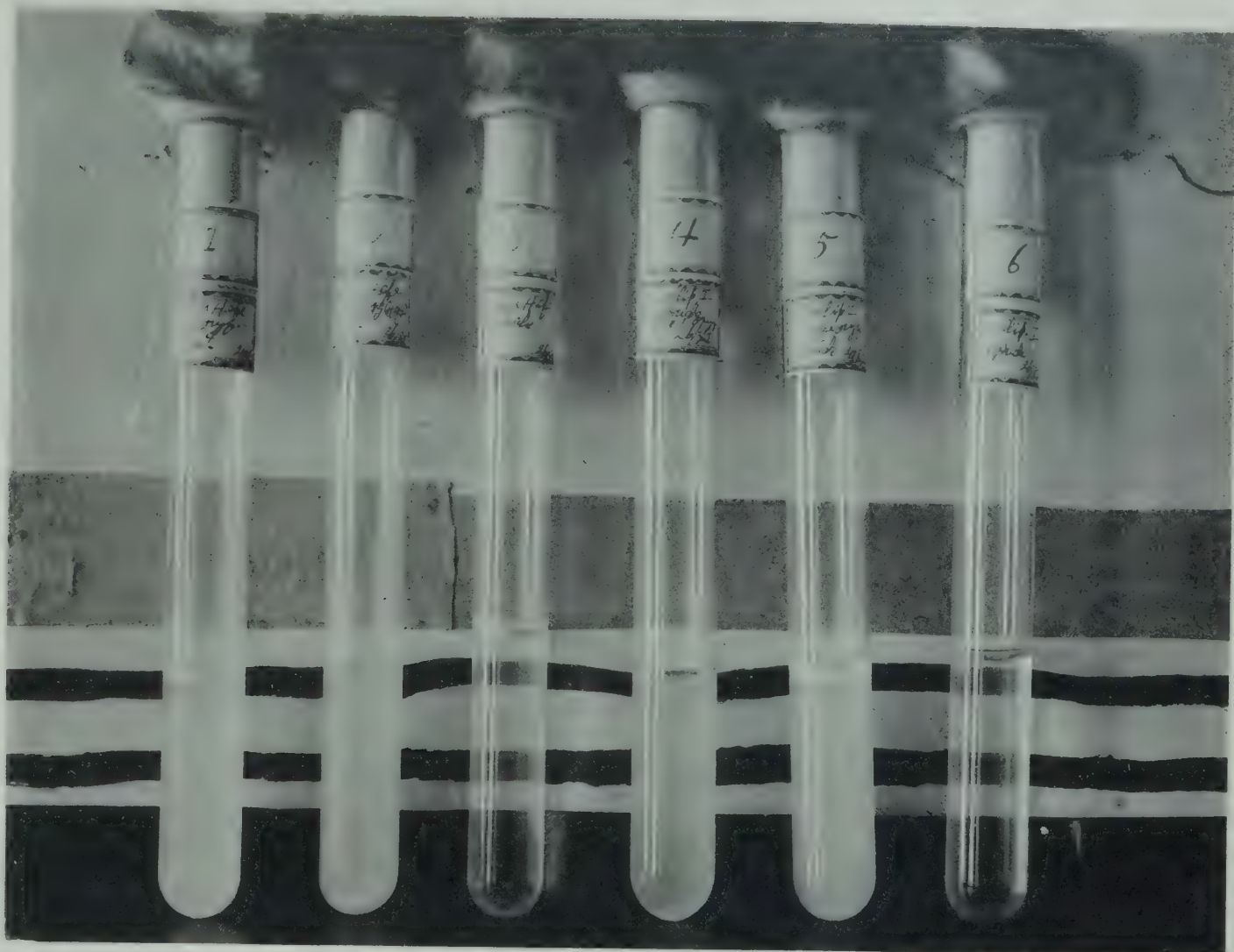
Lösung. Nach zwei Tagen bei 25°C. 1, 4, enthalten 0.01% Kleie-Oryzaninextrakt. 2, 5, enthalten 0.01% Hefe-Oryzaninextrakt. 3, 6, ohne Oryzanin.

Fig. 5. *Saccharomyces cerevisiae*. (A, B, C) und untergärige Bierhefe, Froberg (D, E, F) in Hayduckscher Lösung. Nach zwei Tagen bei 25°C. A, D, enthalten 0.01% Kleie-Oryzaninextrakt. B, E, enthalten 0.01% Hefe-Oryzaninextrakt. C, F, ohne Oryzanin.



3.





On the Canned Crab.

By

Yuzuru Okuda and Hidesaburo Matsui.

The canned crab which is exported from Japan is the flesh of *Paralithodes camtschatica*, chiefly caught off the coasts of Hokkaido and Chishima. It is very tasteful, but sometimes shows a black coloration, on account of which it is disagreeable to eat. To ascertain the cause of this undesirable change and to prevent it, we made some experiments.

About the chemical composition of crab extract, A. RÖHRIG¹ and H. BARSCHALL² made a simple analysis. D. ACKERMANN and FR. KUTSCHER³ found tyrosine, leucine, arginine, lysine, hypoxanthine, betaine, neosine, pyrimidine methyl chloride, flesh lactic acid, crangitine ($C_{13}H_{20}N_2O_4$) and crangonine ($C_{13}H_{26}N_2O_3$), and lately Prof. U. SUZUKI, R. INOUE and K. C. BHARATKAR⁴ isolated arginine, histidine, leucine, tyrosine, alanine and kanirine ($C_6H_{14}N_2O_2$).

Regarding the black substance sometimes found in tinned crab, several years ago T. YOSHIOKA assumed and lately Prof. S. MACHIDA⁵ ascertained it to be iron sulphide. The latter also investigated the cause of the discoloration, and pronounced bacteria as its agent. BUTTENBERG⁶ found that there is some, but no definite relation between the black coloration and the growth of bacteria and quantity of ammonia, and also that all the canned crab experimented on by him showed alkaline reaction.

1. A. RÖHRIG: Zeitschr. f. Unters. d. Nahrungs- u. Genussm. XII, 559.—Orig., Berichte d. chem. Untersuchungsanstalt. Leipzig. 1905, 11.

2. H. BARSCHALL: Jahresb. f. Tierchemie 1910, 482.—Orig., Arb. a. d. kais. Gesundheitsamte, 1909, 30, 74.

3. D. ACKERMANN UND FR. KUTSCHER: Zeitschr. f. Unters. d. Nahr.- u. Genussm. 1906, 13, 180-84; 610-13; 613-14; 1907, 14, 687-91.

4. U. SUZUKI, R. INOUE and K. C. BHARATKAR: Journ. Coll. of Agriculture, Imperial University of Tokyo, V. I, 9, 1912.

5. S. MACHIDA: Journ. Scient. Agricul. Society (Nōgaku Kwai Hō), 108 and 110.

6. BUTTENBERG: Zeits. f. Unters. d. Nahrungs- u. Genussm, 1908, XVI. 92.—1910, XX, 311.

I. Composition.

A. Composition of fresh crab.—Preceding the experiments with the canned flesh of *Paralithodes camtschatica*, we intended to analyze the fresh flesh, but unfortunately we could not obtain it, therefore several allied kinds of flesh were analyzed, and compared with the flesh of fish.

	In per cent of fresh matter.	In per cent of dry matter.		
	<i>Neptunus pela- gicus</i> ¹ M-Edw.	<i>Nept. pelagicus.</i>	<i>Palinurus japo- nicus</i> ¹ Gray.	"Hako-ebi" ² (a kind of lobster)
Water	80.37	0.00	0 00	0.00
Dry matter	19.63	100.00	100.00	100.00
Total N	2 14	10.91	14.81	13.59
Alb. N	1.39	7.09	10.98	10.54
Soluble N	1.14	5.81	6.60	—
Soluble alb. N	0.39	1.99	2.58	—
Non-alb. N	0.75	3.82	4.02	3.05
N in phosphotungstic ppt.	0.15	0.76	2.54	1.58
Ammonium N	—	—	trace	0.38
Mono-amino-acid N	0.03	0.15	0.61	} 1.06
Other N	0.57	2.91	0.87	
Soluble matter	9.28	47.27	—	—

Comparison of soluble nitrogen :

Sample. In per cent of dry matter.	Fresh flesh.									Canned flesh of <i>Paral.</i> <i>camtsch.</i> ³				
	Bonito.	Tunny.	Porgy.	Carp.	Crucian carp.	Cuttle fish.	Spiny lobster.	"Watariguni" (a kind of crab)	"Hakoebi"	Male.	Female.	Black changed.	Washed.	Not washed.
Soluble alb. N	3.09	3.07	2.56	1.84	2.53	2.61	2.59	1.99	—	1.71	1.10	1.17	1.61	1.11
Non-alb. N	2.22	2.71	1.86	1.94	3.24	2.40	4.02	3.82	3.05	1.83	2.96	3.32	3.11	4.77

From the table we see that the quantity of non-albuminous nitrogen in the flesh of Crustacea is generally superior to that in fish.

- 1. Fresh ; amphoteric reaction.
- 2. Left for 24 hours at 0° ; faintly alkaline reaction.
- 3. In canned flesh, there is generally little soluble nitrogen, for the flesh is usually boiled and washed before canning.

B. Analysis of the canned flesh of Paralithodes camtschatica.

a. Qualitative test.—Canned flesh, about one year after preparation, having a reddish violet color and ammoniacal odor, and giving an alkaline reaction, contained iron sulphide, ammonia, amine, tyrosine and trace of nitrate.

b. Influence of acid.—To ascertain the influence of acid upon the flesh, two cans were filled with the same crab, one can being acidified on preparation and the other not, and analyzed simultaneously after one year.

	Acidified.		Not acidified.	
Contents of can	449 gm.		444 gm.	
Quantity of flesh	439 "		436 "	
Appearance	Almost normal, but a few small gray spots were found here and there.		Do.	
Reaction	Alkaline.		Do.	
Odor	Normal.		Do.	
	In per cent of		In per cent of	
	Substance.	Dry matter.	Substance.	Dry matter.
Water	74.51	0.00	74.42	0.00
Dry matter	25.49	100.00	25.58	100.00
Total N	3.52	13.81	3.58	13.99
Alb. N	2.70	10.59	2.77	10.83
Non-alb. N	0.82	3.23	0.81	3.16
Soluble N	0.99	3.88	0.93	3.64
N in phosphotungstic ppt	0.48	1.88	0.36	1.41
Ammonium N	0.06	0.24	0.06	0.23
Organic base N	0.42	1.65	0.30	1.17
Monoamino N	0.13	0.51	0.12	0.47
Other N	0.21	0.82	0.33	1.26
Crude ash	1.72	6.75	1.92	7.51
Soluble matter	7.84	30.76	7.84	30.65
Sulphur	{ black part white part	0.38	0.57	2.23
			0.53	2.07
Iron	{ black part white part	0.008	0.012	0.040
			0.006	0.020
Nitrate	Trace		Trace	
Tyrosine	+		+	
Tryptophane	—		—	

There was no remarkable difference in the composition in both cases, but the gray part of the flesh contained a little more iron¹ and sulphur than the white part, and the most striking fact was that the two gave an alkaline reaction.²

c. The influence of time upon canned flesh.

	In per cent of dry matter.		In per cent of soluble N.	
	9 months old.	2 years old.	9 months old.	2 years old.
Organic matter	94.00	88.53		
Inorganic matter	6.00	11.47		
Total N	10.62	14.71		
Soluble matter	35.26	36.41		
Soluble N	4.72	4.49		
Soluble alb. N	1.62	1.17	34.16	26.12
Non-alb. N	3.11	3.32	65.84	73.88
Organic base N	0.75	0.64	15.88	14.31
Ammonium N	0.27	0.38	5.74	8.47

From this table it seems probable that some decomposition occurs and that the quantity of ammonia increases in time.

d. The influence of the sex of the crab.—Analysis was performed with two cans, one month after preparation.

	In per cent of dry matter.		In per cent of soluble N.	
	Male crab.	Female crab.	Male.	Female.
Organic matter	90.75	89.41		
Inorganic matter	9.25	10.59		
Total N	13.07	13.21		
Soluble matter	29.68	34.41		
Soluble N	3.54	4.06		
Soluble alb. N	1.71	1.10	48.30	27.23
Non-alb. N	1.83	2.96	51.70	72.77
Organic base	1.19	0.66	33.61	16.25
Ammonium N	0.12	0.13	3.39	3.04
Sulphur	1.24—1.62	2.09—2.75		

1. This coincides with the observation of S. MACHIDA.

2. " " " " " " BUTTENBERG.

From the table we see, that female crab contains much more soluble matter, non-albuminous nitrogen and sulphur than the male, therefore the latter is the better of the two for canning.

e. Difference between the black-changed canned flesh and the normal.

Reaction.	Black-changed Somewhat strong alkaline.	Normal. Faintly alkaline.	Normal. Do.
Water	77.90	76.76	78.51
Dry matter	22.10	23.24	21.49
Total N	3.25	3.04	2.84
Soluble mater	8.05	6.90	7.40
Sol. organic matter	6.54	5.18	2.26
Sol. inorg. matter	1.51	1.72	1.14
Soluble N	0.99	0.82	0.87
Soluble alb. N	0.41	0.40	0.24
Non-alb. N	0.58	0.42	0.43
N in phosphotungstic ppt.	0.14	0.04	0.10
Ammonium N	0.09	0.03	0.03
Other N	0.44	0.38	0.53
Iron (Fe)	—	0.0003	0.0014
Iron soluble in 1% Hcl	0.0040	0.0001	0.0002

Sulphur content, in dry matter:—Black part 2.23%; white part 1.49—2.00%.

From these results, identical with the result of (b), we see that the quantities of nonalbuminous nitrogen, ammonia, iron and sulphur in the black-changed flesh are superior to those in white flesh. In another case, where the flesh was sterilized in a glass bottle instead of an iron can, we found the content of iron in the flesh to be similar to that in the normal canned flesh. There must be, therefore, a close relation between the black-changing of the flesh and the imperfection of the iron can.

f. Sulphur content.—The sulphur content in the flesh of Crustacea seems to be somewhat higher than that in fish, for we found 1.24—2.75 per cent sulphur in dried flesh of *Paralithodes camtschatica*, and one of our friends, H. YAMAKAWA, also found respectively 1.34 and 1.41% S in the flesh of a

lobster and a crab, while KÖNIG and SPLITTGERBER¹ obtained 0.71—1.45, average 1.12% S in many kinds of fish.

II. Loss on Boiling and Washing.

Before canning the flesh of the crab is usually boiled and washed. The loss by this process was investigated with the following result :

	Flesh of <i>Paralithodes camtschatica</i> .		Difference.
	Before boiling & washing.	After boiling & washing.	
Water	79.55	77.22	−2.33
Dry matter	20.45	22.78	+2.33
Organic matter	18.93	21.41	+2.48
Inorganic matter	1.52	1.37	−0.15
Total N	2.72	2.42	−0.30
Alb. N	1.75	1.71	−0.04
In soluble matter.			
Dry matter	9.68	8.04	−1.64
Organic matter	8.25	6.94	−1.31
Inorg. matter	1.43	1.10	−0.33
Total N	1.20	1.08	−0.12
Alb. N	0.23	0.37	+0.14
Non-alb. N	0.97	0.71	−0.26
Organic base N	0.31	0.17	−0.14
Other N	0.66	0.60	−0.06
Calculated for dry matter per cent.			
Organic matter	92.58	94.00	+1.42
Inorganic matter	7.42	6.00	−1.42
Total N	13.22	10.62	−2.60
Alb. N	8.45	7.51	−0.94
Soluble matter	47.35	35.26	−12.09
Sol. organic matter	40.35	30.77	−9.58
Sol. inorg. matter	7.00	4.49	−2.51

1. KÖNIG u. SPLITTGERBER: Die Bedeutung der Fischerei für die Fleischversorgung in Deutschland, 1909.

	Before boiling & washing.	After boiling & washing.	Difference.
Sol. Total N	5.88	4.72	-1.16
Sol. alb. N	1.01	1.61	+0.60
Non-alb. N	4.77	3.11	-1.66
Organic base N	1.53	0.75	-0.78
Other N	3.15	2.36	-0.79

III. Autolysis of Crab (*Neptunus Pelagicus* M-Edw.)

To see whether autolysis is going on or not, 70 gm. of fresh flesh, which had an amphoteric reaction, was ground in a mortar and divided into two equal parts, and put into the flasks *A* and *B*. After adding 400 c.c. of water to each flask, *A* was boiled for a few minutes to destroy the enzymatic action. Both flasks were then shaken with enough toluole and chloroform and kept for 3 days at 35°—37°. No bacterial growth was observed during that time. Both flasks were then boiled, filtered and analyzed with the following results :

	In per cent of fresh flesh.	
	Not boiled (<i>B</i>).	Boiled (<i>A</i>).
Soluble total N	1.01	0.99
Soluble alb. N	0.07	0.11
Non-alb. N	0.94	0.88
N in phosphotungstic ppt.	0.33	0.41
Monoamino N	0.11	0.06
Other N	0.50	0.41
N after formol method	0.33	0.22
Ammonia	Little	Very little

The same experiment was repeated with other flesh of *Neptunus pelagicus*. In this case we only determined nitrogen after the formol method, which seemed to us the simplest and most convenient method for the estimation of autolysis. The result was as follows :

Not boiled 0.38 %. Boiled 0.26 %.

We see from the above two simple experiments that autolysis is going on in the fresh flesh of the crab, therefore care must be taken regarding this point in canning.

Tyrosinase. When we exposed some white flesh of *Neptunus pelagicus* to the open air, we observed that it changed into gray. To ascertain whether tyrosinase is present or not, the pressed fluid from the flesh was mixed with an equal volume of 0.05 % tyrosine solution and left for 10 hours, protected from bacteria with some chloroform and toluole. After that time, the mixture turned brown, but the control remained without any change.

In the blood of *Palinurus Japonicus* a stronger tyrosinase was observed by a similar method. The color of the mixture turned orange at first, but it changed after one hour through reddish brown into black.

IV. Change by Boiling.

By merely boiling with water, crab changes remarkably.

A. Change of composition.—Experimented with the fresh flesh of *Neptunus pelagicus*.

Experiment 1.	In per cent of fresh flesh.		Difference.
	Fresh flesh.	Flesh heated in a closed bottle for 2 hours at 100°.	
Soluble matter	9.28	8.28	−1.00
Soluble N	1.14	1.00	−0.14
Sol. alb. N	0.39	0.14	−0.25
Non-alb. N	0.75	0.86	+0.11
N in phosphot. ppt.	0.15	0.35	+0.20
Monoamino N	0.03	0.04	+0.01
Other N	0.57	0.47	−0.10
Sulphide	—	+	
Experiment 2.			
Sol. alb. N	0.43	0.19	−0.24
Non-alb. N	0.67	0.70	+0.03
Monoamino N	0.07	0.09	+0.02
Sulphide	—	+	

From this we see that some chemical decomposition occurs by the mere boiling of crab flesh, and some volatile sulphide and ammonia evolve. A similar change will most likely occur in canning.

B. Evolution of a volatile sulphur compound from flesh by boiling.—This fact was ascertained with several kinds of flesh.

a. *Neptunus pelagicus*.—1. About 50 gm. of the fresh flesh which contained no sulphide, was put into a well closed glass bottle with some water free from sulphide, and boiled for about two hours in a Koch's steam-pot. The bottle was then opened and tested for sulphide, with positive result, by means of a piece of lead acetate paper hung into the bottle over the solution, or by adding a solution of sodium nitroprussate and alkali to a portion of the solution.¹ After this experiment the boiled flesh was separated from the solution and washed with water until no sulphide-reaction appeared, and finally the flesh was boiled once more in the same way. The sulphide-reaction was then again positive.

2. When a piece of polished iron and silver were boiled with some flesh containing no free sulphide, we found that the metals and the flesh, where it came in contact with them, had been colored somewhat black or brown,² and some hydrogen sulphide liberated from the resultant substances by adding a dilute solution of sulphuric acid. Therefore it seems to be a fact that the volatile sulphur compound, freed from flesh by boiling, acts so as to combine with the metal.

b. Canned flesh of *Paralithodes camtschatica*.—It contained some sulphide already. Therefore, to remove this, the flesh was stirred with dilute sulphuric acid solution for about one hour at 30°—40°, filtered, repeatedly washed, and finally neutralized with a drop of dilute alkali. A portion of flesh was directly used for the detection of sulphide, but the test proved negative, while from the other portion, by boiling, volatile sulphur compound was evolved again. When it was boiled with iron, its sulphide was also found.

c. The flesh of horse, hen, carp and yellow-tail, also produced some volatile sulphur compound by boiling.

1. Lead acetate test was more pronounced than the nitroprussate reaction.

2. Iron sometimes makes black oxide by merely boiling with water, but in this case, of course, hydrogen sulphide does not evolve by adding acid.

From these results it appears, that almost all kinds of flesh seem to produce some volatile sulphur compound by boiling with water.

C. Change of reaction.—The fresh flesh of *Neptunus pelagicus* which had amphoteric reaction, and the canned flesh of *Paralithodes camtschatica* which was washed quite well until it gave neutral reaction, were boiled with some water in closed glass bottles for about two hours. In both cases their reaction inclined somewhat towards the alkaline. When the flesh was boiled with some iron powder prepared by Merck, the increase of alkalinity was more remarkable, and this alkaline compound was volatile.

V. Relation between the Iron Casing and the Alkalinity of its Contents.

To study this relation, four cans, two years old, were opened and analyzed with the following results:

No.	Reaction of the canned flesh of <i>Paral. camt.</i>	Appearance.	c.c. of standard H_2SO_4 or NaOH, required for the neutralization of 10 c.c. of the juice in can.	Mgm. of iron in 100 gm. of flesh.
1	Basic	Reddish violet	26 (NaOH)	16.8
2	"	Do., most disagreeable.	28 "	14.0
3	"		15 "	11.5
4	Acid	White, normal.	16 (H_2SO_4)	7.7

From this we see that the stronger the alkalinity in a certain degree of concentration, the more it dissolves iron from the can.

To ascertain this result, we made seven cans of the same size from a sheet of iron, filled them with equal amounts of the flesh of *Paralithodes camtschatica*, and sterilized after the addition of some acid or alkali. These cans were opened and analyzed after half a year.

No.	Amount of acid added.	Iron found in 100 gm. of flesh.
1	0.43 mgm.	2.26 mgm.
2	1.08 "	2.02 "
3	2.17 "	1.59 "
4	4.37 "	0.67 "

No.	Ammonia added.	Ammonia found.	Iron in 100 gm. of flesh.
1	28.3 mgm.	167.4 mgm.	2.11 mgm.
2	56.6 „	215.0 „	2.11 „
3	113.3 „	227.8 „	17.64 „

These results express the same relation as the above experiment, and also show that if some acid is added beforehand, the quantity of iron in the solution is less than otherwise.

Alkaline reaction of crab.—The fresh flesh generally gives amphoteric reaction, but after a few hours it reacts either acid or alkaline. In this case we observed that crab-flesh alone inclined to alkaline reaction, while many other kinds of flesh which we examined, inclined to acid reaction. Not only fresh but also canned flesh has similar relation. We examined the reaction of two or three hundred canned crabs, and found, with BUTTENBERG, almost all of them to be alkaline, while other canned flesh, such as beef, mackerel and bonito, was acid.

VI. On the Cause of the Black-Change of Canned Crab.

We have already proved that the greater part of the black substance in canned crab is iron sulphide, and have also artificially produced the sulphide from iron and flesh by boiling. The causes of the formation of the black substance can be classified as follows:

A. Alkaline reaction. This is an important cause for the black-alteration of the flesh. From our preceding experiments we observed: i. When several kinds of fresh flesh which had amphoteric reaction were allowed to stand for a few hours, their reaction generally turned to acid, in the case of crab-flesh, however, to alkaline. ii. The canned flesh of crab had generally alkaline reaction, while other canned flesh had acid reaction. iii. By boiling, crab-flesh evolved some volatile sulphur compound, as other flesh, but, in contrast to the other, also some volatile alkaline substance. iv. The stronger the alkaline reaction, the more the juice dissolved iron from the wall of the can. These facts show the cause of the black change, namely:

crab-flesh has the characteristic property of making iron sulphide from an iron solution by mere boiling.

B. Action of bacteria. Prof. S. MACHIDA, after experiments, described that the hydrogen sulphide and ammonia, produced from crab by the agency of putrefying bacteria, act on the iron casing and form black iron sulphide. We did not further examine the bacteria, but we found that in comparison with fish, crab is more decomposable, more easily changeable to give alkaline reaction, contains more sulphur, and requires a longer time for sterilization, therefore we believe bacterial action to be a cause of the black change in the case where the sterilization is imperfect.

C. Relation of body-fluid. Tyrosinase, existing in crabs' blood, gives a dirty color to the flesh before canning. Blood contains more sulphur than flesh, being convenient to the formation of sulphide.

D. Ferrous-ferric hydroxide. By boiling with water, iron, in most cases, gives a black colored salt. In the presence of ammonia it is especially liable to form the black double salt, ferrous ferric hydroxide, which will be produced sometimes in the can.

E. Wrapping paper. The quantity of reducing sugar in the paper which is used to envelop the flesh in cans, has some action on the color of the flesh, namely it is colored if there is much sugar.

VII. Summary and Conclusion.

1. The quantities of non-albuminous nitrogen and sulphur in the flesh of Crustacea are generally superior to those of fish.

2. Crabs, as well as many kinds of other flesh, liberate some volatile sulphur compound by boiling with water. This fact suggests the presence of some unstable combination of sulphur in almost all muscle-proteins.

3. A somewhat remarkable autolysis goes on in the fresh flesh of the crab, therefore it is better to boil it immediately after fishing.

4. The female crab contains more sulphur and non-albuminous compounds than the male, consequently, for canning, the flesh of the former is inferior to the latter, both from a chemical and biological point of view.

5. In comparison with other kinds of flesh, crab is more changeable to

alkaline reaction. This is caused not only by autolysis or by bacterial action, but also by merely boiling with water. The last fact is characteristic for crab.

6. Almost all canned crab gives an alkaline reaction, while other canned flesh has an acid reaction. The alkalinity increases step by step with time, the stronger the alkalinity, the more iron is dissolved from the wall of the can.

7. Of soluble organic matter, nonalbuminous compounds, sulphur and ammonia, the black-changed flesh contains a higher quantity than the normal flesh.

8. The black substance in canned crab is chiefly iron sulphide.

9. The cause of the black-change of canned crab must chiefly be attributed to its nature, which is easily changeable to alkaline reaction.

10. We shall be able to avoid the black-changing of the flesh either by carefully selecting the material for the making of the cans or by previously adding some acid to the contents. Care must also be taken to use comparatively fresh flesh, to boil and wash it before preparation, to sterilize it thoroughly, and to carefully select the paper for wrapping it.

We wish to thank Professors U. SUZUKI and S. MACHIDA for their kind advice given during the progress of this work.

On the Quality of “Asakusanori.”

By

Yuzuru Okuda and Seisaku Nakayama.

A Japanese food “Asakusanori” or “Hoshinori” is made of *Porphyra tenera* Kjellm by drying. It has an appearance like coarse black paper and has been known in Japan since remote ages, and at present is still very widely consumed, forming a favorite article of diet for the people.

Only three analytical results are found in NAGAOKA's chemical tables,¹ but no further details. In the present work, therefore, some attempts have been made to ascertain the relation between the quality and the chemical composition of the food.

Exp. 1. Analytical results were as follows:

Sample.	1	2	3
Price (sen) of 100 gm. of dried sample..	74.8	52.8	52.7
In 100 gm. of original sample.			
Dry matter.....	96.44	93.62	93.94
Total N	6.20	5.26	4.60
Protein N,.....	5.05	4.23	3.70
Non-prot. N	1.15	1.03	0.90
Organic base N.....	0.04	0.02	0.03
Monoamino-acid N	0.18	0.22	0.14
Other N	0.93	0.79	0.73
Carbohydrate (as starch)	21.96	19.94	22.23
NaCl (calculated from total chlorine)	2.60	2.53	5.00
In 100 gm. of dried sample			
Total N	6.43	5.62	4.90
Protein N	5.24	4.52	3.94

1. NAGAOKA: Chemical Tables for daily Use, 154.

Exp. 2. Nitrogen content was chiefly examined in the following samples.

Date of the analysis: November 15, 1911.

Name of the shop, from which the sample was obtained: Yamamoto.

Sample:	1	2	3	4	5
Price (sen) of 100 gm. of dried sample	77.6	68.3	67.1	55.5	50.9
In 100 gm. of original sample.					
Dry matter.....	94.31	94.34	93.11	93.99	94.75
Total N	6.91	6.56	6.37	5.86	5.81
Protein N	5.64	5.57	5.14	4.79	4.71
Carbohydrate (as glucose)	24.63	19.35	19.33	15.07	13.69
In 100 gm. of dried sample.					
Total N	7.33	6.95	6.84	6.24	6.13
Protein N	5.98	5.90	5.52	5.10	4.97
Carbohydrate	26.11	20.51	20.77	16.04	14.44

Exp. 3. Date of analysis: January 25, 1912.

Name of shop: Yamagataya.

Sample:	1	2	3	4	5	6	7	8
Price of 100 gm. of dried sample ..	64.0	56.6	54.4	51.4	50.7	50.3	43.0	35.0
In 100 gm. of original sample.								
Dry matter	87.26	87.37	87.64	86.90	82.85	87.12	86.21	85.79
Total N	5.80	5.40	5.45	5.58	4.57	5.44	5.13	3.69
In 100 gm. of dried sample.								
Total N	6.65	6.19	6.21	6.42	5.51	6.24	5.94	4.30
Averaging 2 and 3; 4, 5 and 6, in above results.								

Sample.	1	2 & 3	4, 5 & 6	7	8
Price	64.0	55.5	50.7	43.0	35.0
Nitrogen	6.65	6.20	6.06	5.94	4.30

The results of the above experiments show, that the superior samples generally contain more nitrogen than the inferior ones, and the nitrogen, relating to the quality, belongs chiefly to food protein.

On the Form of Iodine in Marine Algae.

By

Yuzuru Okuda and Toku Eto.

The existence of somewhat remarkable quantities of iodine in some marine algae is a matter of importance both from the physiological and the technical point of view, but as to the form of this iodine there exist only a few investigations. The form of iodine in brown algae was studied by ESCHLE¹ and by TSUKAMOTO and FURUKAWA,² but with opposite results, the former reporting it to be in an organic combination, while the latter two considered it to be inorganic. This disagreement drew our attention to the investigation of the same problem. We have ascertained it to be chiefly organic, and, moreover, have made some further experiments.

Materials employed in course of the experiments were the following: *Ecklonia cava*, *Ecklonia bicyclis* (*Eisenia arborea* f. *bicyclis*), *Turbinaria fusiformis*, *Sargassum enerve* and *Sargassum horneri*.

I. The Chief Form of Iodine in Marine Algae is Organic.

At first we have made some qualitative tests with very fresh extracts of *Ecklonia bicyclis*. They gave no reaction of free iodine, iodate and of periodate. Iodide was sometimes absent and sometimes in trace. But the extract always gave strong iodide reaction after fusing with sodium carbonate and potassium nitrate. From these results we assumed that the iodine is chiefly in an organic combination, and ascertained it by further experiments.

Determination of iodine.—Inorganic or iodide iodine was determined by FRESSENIUS' method.³ Aqueous extract of algae was acidified with sulphuric

1. ESCHLE: Zeitschr. f. physiol. Chem., 23, 30, 1897.

2. TSUKAMOTO and FURUKAWA: Jour. Agric. Soc. 128, 1, 1912.

3. FRESSENIUS' method: Treadwell, Lehrbuch d. analyt. Chem. II, 539, (1911).

acid, filtered from a voluminous precipitate, treated with nitrite and chloroform, and the chloroform layer was titrated with the standard sodium thiosulphate solution. Organic iodine in the aqueous solution separated from the chloroform layer was determined after TSUKAMOTO and FURUKAWA,¹ namely, transforming the organic iodine into iodide form by fusing with sodium hydroxide and potassium nitrate. Iodine in the sulphuric acid-precipitate, and iodine in the insoluble residue were also converted into iodide form and determined.

A. ECKLONIA CAVA.

Exp. 1. 5 gm. of the dried and pulverized sample (moisture 2.5 per cent) and 200 c.c. of water were put in a flask with a reverted cooler, and heated for 2 hours in a boiling water bath. Analytical results for iodine were as follows :

Iodine.	In 100 gm. of dry matter.	Ratio. Total iodine as 100.
Soluble inorganic	0.020	11
Soluble organic	0.150	80
In residue, and in sulphuric acid ppt.	0.017	9
Total	0.187	100

Exp. 2. Similar experiments were repeated with the same sample and the following solvents, instead of water.

Iodine.	Absolute alcohol	95 per cent alcohol	2 per cent KOH	2 per cent H ₂ SO ₄
Sol. inorganic (in 100 gm. of dry matter)	0.009	Trace	Trace	0.012
Sol. organic („ „ „ „ „ „)	0.080	0.140	0.174	0.171
Sol. inorganic (total iodine as 100)	5	Trace	Trace	6
Sol. organic („ „ „ „)	43	75	93	92

Thus, over 90 per cent of the total iodine of this algae is in a soluble organic form, which is not decomposed by boiling with dilute sulphuric acid or potassium hydroxide.

1. TSUKAMOTO-FURUKAWA's method is a modification of BAUMANN—Roos' method (Zeitschr. f. physiol. Chem., XXI, 489, 1895).

B. ECKLONIA BICYCLIS.

Exp. 1. Sample 1 (obtained in December, 1913, at Misaki). Immediately steamed after sampling, and dried and powdered the next morning.

In hot water extract.	In 100 gm. of dry matter.	Total iodine as 100.
<i>A.</i> I in sulphuric acid precipitate	0.007	4
<i>B.</i> Inorganic I, in the filtrate of <i>A</i>	0.004	3
<i>C.</i> I in absolute alcohol ppt., in the filtrate of <i>B</i>	0.004	3
<i>D.</i> Organic I, in the filtrate of <i>C</i>	0.127	82
<i>E.</i> Soluble total I	0.142	92
In residue		
<i>F.</i> I soluble in 3 per cent KOH	Trace	—
<i>G.</i> I in the last residue	0.013	8
Total iodine	0.155	100

Sample 2. (January, 1914, at Misaki). Dried, powdered and extracted with boiling water, as in the above experiments.

Iodine.	In 100 gm. of dry matter.	Total iodine as 100.
In sulphuric acid ppt.	0.033	19
Soluble inorganic	0.000	0
Soluble organic	0.130	73
In residue	0.015	8
Total	0.178	100

Exp. 2. Sample 3. (May, 1914, at Enoshima).

It was divided into three portions, *A*, *B* and *C*.

A. Dried and powdered several hours after sampling.

B. Extracted, without drying, several hours after sampling.

C. Dried and powdered after preserving for several hours in chloroform.

Iodine.	A (dried).		B (fresh).		C (dried with chloroform).	
	In 100 gm. of dry matter.	0.202 as 100.	In 100 gm. of dry matter.	0.202 as 100.	In 100 gm. of dry matter.	0.164 as 100.
Sol. inorganic	0.013	6	0.003	2	0.036	22
Sol. organic	0.172	85	0.193	96	0.105	64
In residue and sulphuric acid ppt.	0.017	9	—	—	0.025	14
Total	0.202	100	—	—	0.164	100

Thus, the amount of soluble organic iodine diminishes by drying at 100° C, and the decrease is more remarkable on drying with chloroform. In the latter case, also, some iodine seems to be volatilized.

Exp. 3. Sample 4. (June, 1914, at Enoshima). Dried and powdered sample was extracted with cold water with a little chloroform for 24 hours.

Iodine.	In 100 gm. of dry matter.	Total iodine as 100.
Sol. inorganic	0.059	17
Sol. organic	0.146	42
Total	0.348	100

Exp. 4. Sample 1. Extracted with 95 per cent alcohol.

	In 100 gm. of dry matter.	Total iodine as 100.
Water sol. inorg. I	0.0	—
I in water insol. residue	Trace	—
Water sol. org. I	0.140	90
I in alcohol insoluble residue	0.015	10

From all the above experiments we see, that fresh samples of this algae contain only trace — 4 per cent of iodine in iodide form, the largest part being soluble organic iodine, and also, that the quantity of iodine in the algae increases from winter to summer.

C. TURBINARIA FUSIFORMIS YENDO, SARGASSUM ENERVE AG. AND
SARGASSUM HORNERI AG.

Some decomposition took place in these samples, owing to their being preserved in chloroform for one day, and then dried at 100°, but the larger part of iodine still remains in organic combination, as the following results show.

Algae.	Sarg. enerve.		Sarg. horneri.		Sarg. enerve.		Turb. fusiformis.	
Time & place.	Feb., Kanazawa.		March, Misaki.		May, Enoshima.		May, Enoshima.	
Iodine.	In 100g. of dry matter.	Total I as 100.	In 100g. of dry matter.	Total I as 100.	In 100g. of dry matter.	Total I as 100.	In 100g. of dry matter.	Total I as 100.
Sol. inorganic	0.024	16	0.010	12	0.017	17	0.013	22
Sol. organic	0.118	78	0.053	66	0.067	66	0.030	50
I in H ₂ SO ₄ ppt.	0.001	1	trace	—	—	—	—	—
I in residue	0.007	5	0.018	22	0.017	17	0.017	28
Total I	0.150	100	0.081	100	0.101	100	0.060	100

II. The Chief Form of Iodine in “Dashikombu”
is Inorganic.

“Dashikombu” is a Japanese food prepared from *Laminaria* by drying and by some fermentation. We extracted a sample with the following solvents by boiling.

Solvent.	Water.		2% H ₂ SO ₄		95% Alcohol.	
Iodine.	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.
Soluble inorganic	0.361	91	0.386	97	0.374	94
Soluble organic	0.012	3	0.007	2	0.017	4
In residue	0.025	6	—	—	—	—
Total	0.398	100	—	—	—	—

Thus, about 95 per cent of total iodine of the sample is in the iodide form.

This result exactly harmonizes with that of TSUKAMOTO and FURUKAWA, but the knowledge obtained from the other experiments leads us to the presumption, that the chief form of iodine in fresh *Laminaria* should be organic, agreeing with ESCHLE's conclusion. We are inclined to attribute this change—from organic to inorganic—to the action of some microorganisms.

III. Action of Enzyme and Microorganisms.

To see whether an enzymatic process or bacterial action plays the greater rôle in the disintegration of the organic iodine compound, we have made some experiments with fresh *Ecklonia bicyclis*.

Exp. 1. Autolysis. 60 gm. of the paste, obtained from the fresh algae by grinding, was equally divided into two flasks, one of which was boiled. To both a little chloroform was added, stoppered and kept for 3 days at about 35°.

Boiled flask. Iodide. Absence or trace.

Unboiled flask. „ Trace.

Exp. 2. Microorganisms. To one of the two flasks, containing the algae and water, a little chloroform was added. Both were stoppered with cotton and kept for two days at 35°.

With antiseptics. Iodide. Trace.

Without „ „ „

Exp. 3. Action of microorganisms and autolysis. 120 gm. of the algae and 1200 c.c. of water were equally divided into 6 flasks, and treated as follows:—

Samples.	Antiseptics.	Remarks.	Iodide iodine.	
			3 days at ca. 35°	6 days at ca. 35°
A. Boiled	Antiseptics were added	Bacterial and enzymatic actions were arrested.	0.0	0.0
B. Unboiled	Do.	Only bacterial action was arrested.	0.0	0.0
C. Unboiled	No addition		Trace	0.004

From these results we see, that the organic iodine compound remains

intact during the autolysis, but undergoes gradual disintegration by the action of some microorganisms.¹

IV. Action of Chlorine Compounds.

Experiments were performed with *Ecklonia bicyclis* containing trace of inorganic iodine.

(a) Sodium chloride. Dilute salt solutions, such as sea water, have a strong decomposing power upon the organic iodine compound, as the following three experiments show.

Experiment.	Sample employed.	3.5% NaCl added.	Treatment.
No. 1.	5 gm.	100 c.c.	Extracted 3 days at room temperature.
No. 2.	„	„	Do., with 2 c.c. of chloroform.
No. 3.	„	200 c.c.	Boiled for 2 hours.

Results.

Experiment.	No. I (cold extract).		No. II (cold extract).		No. III (hot extract).	
	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.
Sol. inorganic	0.084	51	—	—	0.086	52
Sol. organic	0.029	18	0.025	16	0.037	23
Total	0.163	100	—	—	—	—

(b) Calcium chloride. 5 gm. of a sample was extracted with 200 c.c. of 5 per cent calcium chloride solution, for 2 hours in boiling. Inorganic iodine found in the extract was 37 per cent of total iodine.

(c) Algae were boiled with 2.4 per cent hydrochloric acid.

Sol. inorg. I	In 100 gm. of dry matter	0.081	Total iodine as 100	23
Sol. org. I	„	0.135	„	39

(d) Chloroform. Exp. 1. Fresh algae, divided into two portions. *A* was dried at 100°. *B* was dried at 100° with chloroform.

1. We have made some experiments, showing that the organic iodine compound in *Laminaria*, contrary to that of *Ecklonia*, decomposes very rapidly by the action of some microorganisms as well as that of some chemicals.

Sample.	A (no addition).		B (with chloroform).	
	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.
Sol. inorganic	0.013	6	0.036	22
Sol. organic	0.172	85	0.105	64
Total	0.202	100	0.164	100

Exp. 2. Dried sample. A sample was divided equally into four portions, *C*, *D*, *E* and *F*. Total iodine was determined in *C* directly, in *D* after standing for 3 days at room temperature with chloroform, in *E* after heating for several days at 100°, and in *F* after heating for several days with chloroform. The results in *C*, *D* and *E* were practically equal, while in *F* somewhat less.

Thus, chlorides, generally, have a power converting the organic iodine compound into iodide form, probably substituting its chlorine with iodine. Chloroform has also some influence, especially upon fresh algae.

V. Action of Acid and Alkali.

The organic iodine compound in *Ecklonia bicyclis* does not undergo any decomposition in dilute solutions of sulphuric acid or sodium hydroxide, but decomposes completely in concentrated solutions. In a concentrated sulphuric acid solution the largest part of the iodine is freed.

Exp. 1. The mixture of 10 gm. of dried *Ecklonia bicyclis*, 30 c.c. of concentrated sulphuric acid, and 60 c.c. of water was boiled for 16 hours.

Exp. 2. 175 c.c. of the extract of the algae was mixed with 53 gm. of baryta, and boiled for 16 hours.

Iodine.	Exp. 1.		Exp. 2.	
	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.
Sol. inorganic	0.005	1.4	0.256	67
Sol. organic	0.009	2.6	0.0	—
In residue	0.013	3.7	—	—

VI. Action of Formalin.

The organic iodine compound is decomposed most violently by formalin.

Exp. 1. The following four species of algae were kept with a little formalin for 1 day after collecting, then dried at 100° and powdered. After analysis, the larger part of the iodine was found to be inorganic.

Algae.	<i>Ecklonia cava.</i>		<i>Sarg. enerve.</i>		<i>Turb. fusiformis.</i>		<i>Ecklonia bicyclis.</i>	
Time & place.	March, Misaki.		Do.		Do.		Do.	
Iodine.	In 100g. of dry matter.	Total I as 100.	In 100g. of dry matter.	Total I as 100.	In 100g. of dry matter.	Total I as 100.	In 100g. of dry matter.	Total I as 100.
Sol. inorganic	0.146	79	0.047	67	0.065	64	0.160	80
Sol. organic	0.017	9	0.016	23	0.014	14	0.013	6
In H ₂ SO ₄ ppt.	0.006	3	0.003	4	0.002	2	0.002	1
In residue	0.016	9	0.004	6	0.020	20	0.027	13
Total	0.185	100	0.070	100	0.101	100	0.202	100

Exp. 2. The experiment was repeated with fresh *Ecklonia bicyclis* as follows: *A* was kept with a little formalin for 1 day, then dried and analyzed. *B* was kept without formalin and treated as *A*.

Algae.	<i>B</i> (no addition).		<i>A</i> (with formalin).	
Iodine.	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.
Sol. inorganic	0.013	6	0.131	82
Sol. organic	0.172	85	0.015	9
Total	0.202	100	0.161	100

Thus, by drying at 100° with formalin, iodine in the fresh algae changes its form and part of it is lost. The same result was obtained from the analysis of fresh *Sargassum horneri* and *enerve*.

Exp. 3. Dried algae, like the fresh, are attacked by formalin, in acid reaction as well as in neutral, as the following experiments show.

Exp. (a). 5 gm. of dried *Ecklonia bicyclis* with 1 c.c. of formalin was

kept for 3 days at room temperature, and then dried at 100°. Only inorganic iodine was determined with the following results.

Acidic formalin added.		Neutral formalin added.		No addition. Control.	
In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.	In 100 gm. of dry matter.	Total I as 100.
0.179	89	0.187	93	0.013	6

Exp. (b). Another sample was treated as in experiment (a), and only total iodine was determined. The per cent in control case was 0.348, while in formalin-sample 0.291.

Exp. 4. 5 gm. of sample was boiled with 200 c.c. of 2 per cent formalin solution for 2 hours. According to the analytical results, all the soluble organic iodine was converted into inorganic form. Boiling with formalin seems to be a simple method for the detection of organic iodine compound in algae.

From the above experiment we see, when the fresh or dried *Ecklonia bicyclis* is heated with formalin, that its iodine not only changes the form, organic to inorganic, but also decreases in quantity.

VIII. Precipitation of the Iodine Compound.

The soluble organic iodine of *Ecklonia bicyclis* is precipitated as protein by basic lead acetate or by STUTZER's reagent.

Exp. 1. The hot water extract of the algae was precipitated by basic lead acetate, and the precipitate decomposed by means of sulphuric acid. In the filtrate of lead sulphate, 78 per cent of total iodine remained as organic iodine, and inorganic iodine was trace. The filtrate of the precipitate of basic lead acetate also contained trace of iodine.

Exp. 2. The hot water extract was subjected to STUTZER's method. The copper precipitate produced, calculating for 100 gm. of dry algae, contained 0.19 gm. of nitrogen and 0.25 gm. of iodine, or 72 per cent of total iodine. The filtrate of the copper precipitate contained only trace of iodine.

IX. The Organic Iodine Compound is not a Protein.

Some of the organic iodine compounds found hitherto in plants and animals are in a stable combination with protein complex, but the chief organic iodine compound in the extract of *Ecklonia bicyclis* is not combined with protein. Whether or not it was split from a protein only by drying and boiling can not be ascertained as yet with certainty. The greater part of the organic iodine is precipitated by basic lead acetate and by copper hydroxide, but it is distinguished from protein by the following properties.

1. It dissolves in hot water, concentrated alcohol and dilute sulphuric acid.

2. The hot water extract was neither precipitated by boiling with a few drops of acetic acid, by picric acid nor by tannic acid in the presence of acetic acid.

3. The extract gave precipitates either by concentrated mineral acid, by sulphuric and phosphotungstic acids, or by the saturation of the extract with zinc sulphate, but every precipitate contained only very little or no iodine.

4. The algae were extracted with 0.2 per cent potassium hydroxide and the extract was precipitated with two times its volume of absolute alcohol, after acidifying with acetic acid,¹ but the precipitate contained only trace of iodine.

X. Summary and Conclusion.

1. Most of the iodine in all the algae examined is in organic combination, as ESCHLE concluded. These fresh algae contain neither iodate nor periodate, but trace or a very small quantity of iodide.

2. The chief organic iodine compound is soluble in water, concentrated alcohol, dilute alkali or acid solution. It differs from a protein.

3. The distribution of iodine in *Ecklonia bicyclis* is shown, for the sake of simplicity, in the following round numbers.

1. The alkaline extract was not precipitated in the usual manner with only acetic acid.

Total iodine as 100.	{	inorganic (soluble) under 5%.	
		organic . . 95%.	
(Extracted with hot water).	{	insoluble (protein ?) 5%.	
		soluble 90 % { protein (?) 5%.	
		{ non-protein 85%.	
		(chief iodine)	

4. About 95 per cent of the iodine in "Dashikombu," prepared from *Laminaria*, is in an inorganic form, as TSUKAMOTO and FURUKAWA found, but we presume that the chief form of iodine in fresh *Laminaria* will be organic.

5. *Ecklonia bicyclis* has no enzyme which splits the organic iodine compound. The compound undergoes gradual disintegration by some micro-organisms.

6. Dilute solutions of sodium chloride, calcium chloride and hydrochloric acid have a strong effect on the decomposition of the iodine compound, and chloroform also has some influence.

7. Dilute solutions of sulphuric acid and sodium hydroxide have no effect upon the iodine compound of *Ecklonia bicyclis*, but the concentrated solutions decompose it completely.

8. When *Ecklonia bicyclis* is heated with formalin, its iodine not only changes the form, but also decreases the quantity.

9. The organic iodine of *Ecklonia bicyclis* is not decomposed by nascent hydrogen, and is not precipitated by phosphotungstic acid (in distinction from iodogorgonic acid and iodothyrene).

10. Old algae contain more iodine than the young. Total iodine of *Ecklonia bicyclis* increased from winter to summer as follows: December, Misaki, 0.155; January, Misaki, 0.178; March, Misaki 0.202; May, Enoshima, 0.202, and June, Enoshima, 0.348.

11. Generally, algae in an open sea seem to contain more iodine than the same species in an inland sea. The iodine content of the samples obtained in March at Misaki (open sea) was nearly equal to those obtained in May at Enoshima, as follows:

	Iodine in 100 gm. of dry algae.			
	<i>Eckl. cava.</i>	<i>Eckl. bicyclis.</i>	<i>Sarg. enerve.</i>	<i>Turb. fusiformis.</i>
Misaki, March	0.185	0.202	0.070	0.101
Enoshima, May	0.187	0.202	0.101	0.060

12. In dead algae, the largest part of their iodine easily diffuses in sea or fresh water, therefore drifted algae are not suitable for the preparation of iodine.

Hydrolysis of Fish-Gelatine.

By

Yuzuru Okuda.

In physical properties, fish-gelatine somewhat differs from the commercial gelatine which is chiefly obtained from the bone of cattle, but there is no investigation as to whether they differ in the chemical composition or not. Therefore, to contribute something on this point, the writer hydrolyzed a fish-gelatine with hydrochloric acid, baryta, and sulphuric acid, and compared the cleavage products in each case with those of the commercial gelatine already studied by several authors.

The fish-gelatine experimented with was prepared in the Tokyo Imperial Fishery Institute from the skin of the shark, chiefly caught off the coast of Odawara in the province of Sagami.

I. Distribution of Nitrogen.

30 gm. of the fish gelatine and the best commercial gelatine were boiled with three times their weight of concentrated hydrochloric acid for ten hours and analyzed with the following results.

	Fish-gelatine.		Commercial gelatine.	
	In 100 gm. of air dry subst.	In 100 gm. of dry matter.	In 100 gm. of air dry subst.	In 100 gm. of dry matter.
Water	15.33	0.00	14.56	0.00
Total nitrogen	15.02	17.74	14.84	17.38
Of which :				
In hot HCl soluble N	14.67	17.33	14.29	16.73
„ „ „ insoluble N	0.35	0.41	0.55	0.65
Ammonium N	0.31	0.37	0.09	0.11
Organic base N	2.47	2.92	2.52	2.95
Monoamino-acid N	9.69	11.44	9.24	10.82
Other N	2.20	2.60	2.44	2.86
Ash	0.93	1.08	1.45	1.70

Total N as 100.

In hot HCl soluble N	97.68	96.26
Of which : Amm. N	2.09	0.63
Organic base N	16.46	16.97
Monoamino-acid N	64.49	62.25
Other N	14.64	16.45
In hot HCl insoluble N	2.32	3.74

From the table we see a fair agreement in the distribution of the nitrogen of the two, but the quantity of monoamino-acid nitrogen differs somewhat, being superior in the case of the fish gelatine.

II. Monoamino-acids of Fish-Gelatine.

Two portions of the air-dry fish-gelatine (moisture 15.33%, crude ash 0.95%), weighing respectively 450 and 550 gm., were separately hydrolyzed with three times their weight of hydrochloric acid, sp. gr. 1.19, by warming on the water bath and then by boiling for eight hours in an oil bath. The solution was concentrated to a syrup at a low temperature under greatly diminished pressure, and saturated with dry hydrochloric acid gas, but no glutaminic acid has been separated, therefore the syrup was four times treated with absolute alcohol and dry hydrochloric acid gas, and from the whole solution 334.4 gm. of glycocoll ethylester hydrochloride was obtained, melting at 143°—144° and giving the following analytical results :

0.1675 gm. subst. gave 14.6 c.c. N (19.5°, 751 m.m.)
 0.4210 gm. „ „ 0.1071 gm. Cl.

	N	Cl
Calculated for $\text{HCl} \cdot \text{NH}_2 \cdot \text{CH}_2 \cdot \text{COOC}_2\text{H}_5$	10.04	25.40
Found	10.06	25.44

The mother liquor from the hydrochloride of glycocoll ethylester was subjected to esterification in the usual manner. After removing the ether, by distilling on a water bath at ordinary pressure, the esters were distilled under low pressures with the following result :

Fraction.	Temperature of bath.	Pressure.	Weight.
I.	0°—50°	8—13 m.m.	38.6 gm.
II.	50°—80°	" " " "	64.3 "
III.	80°—100°	" " " "	59.2 "
IV.	0°—120°	8—6 " "	42.0 "
V.	120°—180°	4—6 " "	77.7 "
Total			279.8 "
Undistilled residue			53.0 "

Fraction I.—This fraction was saponified by boiling for eight hours with ten times its volume of water. By the evaporation of the solution 4 gm. of alanine crystallized out, melting at 290° and having the following composition :

0.1855 gm. subst. gave	24.5 c.c. N (17° C, 760 m.m.)		
0.1433 gm. " "	0.2116 gm. CO ₂ , 0.1048 gm. H ₂ O.		
	N	C	H
Calculated for C ₃ H ₇ O ₂ N	15.73	40.42	7.93
Found	15.55	40.27	8.19

From the filtrate of alanine by the treatment with the alcoholic solution of picric acid 3.5 gm. of glycocoll picrate was obtained. The crystal decomposed at 200°.

0.1522 gm. subst. gave	23.7 c.c. N (20° C, 764 m.m.)	
	N	
Calculated for C ₆ H ₃ O ₇ N ₃ ·NH ₂ ·CH ₂ ·COOH	18.43	
Found	18.25	

From the filtrate of glycocoll picrate, 2.5 gm. of free alanine melting at 281° was obtained after the removal of picric acid by means of sulphuric acid and ether.

Fraction II.—After saponifying by boiling with water, the solution was evaporated to dryness, and proline was five times extracted by boiling with absolute alcohol. The alcoholic solution, after the removal of other amino acids, left on evaporation 12 gm. of crude proline, which was coupled with freshly precipitated copper hydroxide and the copper salt extracted with absolute alcohol to separate racemic proline from active. By such a treatment 4 gm. of d, l-proline copper was obtained and analyzed with the following result after drying at 110°.

0.2564 gm. subst. gave	20 c.c. N (16° C, 756 m.m.)
	N
Calculated for $C_{10}H_{16}O_4N_2Cu$	9.60
Found	9.16

By means of ice acetic acid and picric acid, l-proline copper was converted into the picrate, which crystallized in the characteristic prisms, melting at 130°. The yield of the picrate was 6.2 gm. and the analytical result was as following:

0.2154 gm. subst. gave	29 c.c. N (19° C, 767 m.m.)
0.7354 „ „ „	0.4884 gm. picric acid.
	N Picric acid
Calculated for $C_{11}H_{12}N_4O_9$	16.29 66.56
Found	15.90 66.41

The amino-acids insoluble in alcohol were dissolved in water and separated into six fractions, weighing respectively 2.0, 4.5, 8.5, 17.6, 7.1 and 13.5 gm. and in each the taste, the melting point and the form of crystals were observed and nitrogen content determined. The first two were practically pure leucine, therefore they were united, recrystallized and analyzed with the following analytical data. The melting point was 299°—302°.

0.1764 gm. subst. gave	16 c.c. N (20 C°, 762 m.m.)
0.1719 „ „ „	0.3442 gm. CO ₂ , 0.1666 gm. H ₂ O.
	N C H
Calculated for $C_6H_{13}O_2N$	10.69 54.97 9.99
Found	10.59 54.61 10.76

The third, fourth, and fifth portions, in union with the last part of the Fraction III, were converted into copper salts, and separated again to eleven fractions, in which the inseparable mixture of leucine and valine was boiled with methyl alcohol in order to separate them from each other, and 45.4 gm. of alanine copper, 0.3 gm. of valine copper and 1.8 gm. of leucine copper were obtained. The alanine copper gave the following analysis:

0.4902 gm. subst. gave	0.1598 gm. Cu O
0.7826 „ „ „	0.0866 „ N
	Cu N
Calculated for $(C_3H_5NO_2)_2Cu$	26.52 11.69

Found 26.04 11.07

The larger part of the last portion of Fraction II was alanine, containing some valine and little glycocoll.

Fraction III.—This fraction was saponified and 11 gm. of crude proline removed as from Fraction II. From the part insoluble in alcohol, after separating and investigating by a systematic fractional crystallization, 21 gm. of leucine and 7.7 gm. of crude valine, containing some leucine, were obtained.

The free leucine, melting at 310° — 315° was analysed as follows:

0.2041 gm. subst. gave 19 c.c. N (18° C, 752 m.m.)

0.2269 „ „ „ 0.4542 gm. CO₂, 0.1902 gm. H₂O

	N	C	H
Calculated for C ₆ H ₁₃ O ₂ N	10.68	54.97	9.99
Found	10.80	54.59	9.32

Fraction IV.—This fraction was saponified with baryta, and 2 gm. of aspartic acid, melting at 280° , was separated. From the filtrate of the barium aspartate, by removing the barium, evaporating to a small volume, and saturating the solution with dry hydrochloric acid gas, 7.4 gm. of glutaminic acid hydrochloride, melting at 194° — 197° , was obtained.

Fraction V.—Phenylalanine ester, separated oily by the addition of water, by shaking with ether and evaporating with concentrated hydrochloric acid, gave 21 gm. phenylalanine hydrochloride, which after recrystallization, melted at 245° — 246° and had the following composition:

0.1702 gm. subst. gave 9.8 c.c. N (18° C, 768 m.m.)

0.0964 „ „ „ 0.0174 gm. Cl.

	N	Cl
Calculated for C ₉ H ₁₁ O ₂ N · HCl	6.95	17.58
Found	6.83	18.00

A portion of this compound was transformed into free phenylalanine and analyzed with the following data:

0.2524 gm. subst. gave 19.2 c.c. N (22° C, 756 m.m.)

	N
Calculated for C ₉ H ₁₁ O ₂ N	8.49
Found	8.75

The aqueous solution, separated from the etherial layer, when saponified

and treated as in the case of Fraction IV, yielded 10.3 gm. aspartic acid and 21.2 gm. of glutaminic acid hydrochloride.

The aspartic acid :

0.2210 gm. subst. gave	18.8 c.c. N (18 C°, 762 m.m.)	
		N
Calculated for $C_4H_7NO_4$		10.53
Found		10.01

The glutaminic acid hydrochloride decomposed at 198°—200° and gave the following analysis :

0.2390 gm. subst. gave	16.1 c.c. N (19 C°, 754 m.m.)	
0.2701 „ „ „	0.0531 gm. Cl	
		N Cl
Calculated for $C_5H_{10}O_4NCl$	7.63	19.31
Found	7.82	19.66

From the filtrate of glutaminic acid, united with the corresponding part of Fraction IV., 6.4 gm. of aspartic acid was obtained by evaporating off the greater part of the hydrochloric acid, boiling with lead oxide, filtering, precipitating the lead with hydrogen sulphide and finally recrystallizing. The substance thus separated had the m.p. of 275°—277°.

0.1338 gm. subst. gave	11.7 c.c. N (19 C°, 744 m.m.)	
		N
Calculated for $C_4H_7NO_4$		10.53
Found		10.01

The filtrate of aspartic acid was treated with β -naphthalin-sulpho-chloride and sodium hydroxide, and 1.8 gm. of β -naphthalin-sulphonate of serine was obtained. But the content of serine must be far more, for I lost a large part of it. This substance melted at 150° and had the following composition :

0.2339 gm. subst. gave	9.5 c.c. N (24 C°, 757 m.m.)	
		N
Calculated for $C_{13}H_{13}O_5NS$		4.75
Found		4.65

III. Determination of Proline.

From 100 gm. air-dry fish-gelatine, by the baryta method after E. FISCHER and R. BOEHNER,¹ 9.2 gm. of racemic proline copper, equivalent to 7.62 per cent proline when calculated for moisture free substance, was obtained.

The analysis of the copper salt was as follows:

0.5250 gm. subst. gave	0.0574 gm. H ₂ O
	H ₂ O
Calculated for C ₁₀ H ₁₆ O ₄ N ₂ Cu + 2 H ₂ O	10.99
Found	10.93

When dried at 100° in vacuum,

0.4676 gm. subst. gave	0.1272 gm. Cu O
	Cu
Calculated for C ₁₀ H ₁₆ O ₄ N ₂ Cu	21.79
Found	21.73

A portion of the racemic salt was converted into the picrate, melting at 128°—130° and having the following composition:

0.2852 gm. subst. gave	41 c.c. N (24.5 C°, 757 m.m.)
	N
Calculated for C ₁₁ H ₁₂ N ₄ O ₉	16.29
Found	16.43

IV. Determination and Isolation of Diamino-acids.

30 gm. air-dry fish-gelatine was boiled with sulphuric acid and examined according to A. KOSSEL's method, described by F. Weiss.²

Result of determination.

	In 100 gm. air-dry subst.		In 100 gm. dry matter.
	N found.	Base calculated.	Base calculated.
Arginine	1.77	5.52	6.53
Histidine	0.17	0.64	0.75
Lysine	0.52	2.74	3.23

1. E. FISCHER u. BOEHNER: Zeitschr. f. physiol. Chem., 65, 118, 1910.

2. F. WEISS: Zeitschr. f. physiol. Chem., 52, 108, 1907.

Arginine was identified as the picrate, which melted at 220°—222°.

0.2074 gm. subst. gave	0.1188 gm. picric acid.
	Picric acid.
Calculated for $C_6H_{14}N_4O_2 \cdot C_6H_3N_3O_7$	56.81
Found	56.68

Histidine was analyzed as the dichloride.

0.1476 gm. subst. gave	0.0461 gm. Cl
	Cl
Calculated for $C_6H_9N_3O_2 \cdot 2HCl$	31.14
Found	31.25

Lysine was converted into the picrate, which melted at 253°—254° and gave the following analysis :

0.1550 gm. subst. gave	0.0935 gm. picric acid.
	Picric acid.
Calculated for $C_6H_{14}O_2N_2 \cdot C_6H_2(NO_2)_3OH$	61.07
Found	60.32

V. Comparison of Decomposition Products.

The decomposition products of the fish-gelatine thus obtained were compared with those of the commercial gelatine, already described by other authors.

Hydrolysis with hydrochloric acid.	Fish-gelatine.	Commercial gelatine.
Glycocoll	21.25	16.50
Alanine	2.83	0.80
Valine.....	1.42 (containing some leucine)	1.00
Leucine	3.43	2.10
Proline	2.72	5.20
Phenylalanine	1.89	0.40
Glutaminic acid	3.07	0.88
Aspartic acid	2.10	0.56
Serine.....	0.12	0.40 ²
Oxyproline.....	—	3.00 ³

1. E. FISCHER, P. A. Levene u. Aders: Zeitschr. f. physiol. Chem. 35, 70, 1902.
2. E. FISCHER u. E. Abderhalden: " " " " 42, 540, 1904.
3. E. FISCHER: " " " " 35, 221, 1902.

Hydrolysis with sulphuric acid.

Lysine	3.23	2.75
Arginine.....	6.53	7.62
Histidine	0.75	0.40

}¹

Hydrolysis with baryta.

Proline	7.62	7.60 ²
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VI. Summaries.

1. As regards the distribution of nitrogen, I did not find any great difference between the fish-gelatine and the commercial gelatine, although some disagreement was to be seen in the content of monoamino-acid.

2. The yields of glycocoll, alanine, leucine, phenylalanine, glutaminic and aspartic acids from the fish-gelatine were much more than those from the commercial gelatine, of which the esters of amino-acids were distilled by higher pressure than in the former case. The amount of proline and serine isolated from the fish gelatine by the ester method was little, but this must be ascribed to an experimental error, indeed, I found the quantity of proline to be equal in both cases after the baryta method.

3. The contents of diamino-acids were approximately equal in the two gelatines.

In conclusion, I wish to express my thanks to Prof. Dr. U. SUZUKI for valuable advice, to Mr. S. KITAKÓJI for kind assistance, and to Mr. K. SIMO for his kindness in supplying material.

1. E. HART: Zeitschr. f. physiol. Chem. 33, 347, 1901.

2. E. FISCHER u. R. BOEHNER: „ „ 65, 118, 1910.

Hydrolysis of Fish Muscle.

By

Yuzuru Okuda and Kiyoshi Ōyama.

The only data relating to the composition of fish muscles are furnished by one hydrolysis of the muscle of halibut made by OSBORNE and HEYL,¹ and by determinations of bases in the muscle of salmon by WEISS.² The question, what constituents and composition distinguish one fish from another, remains unsolved yet. We have accordingly hydrolyzed the muscle substance of *Pagrus major*, one of the most common fish of Japan, to learn its composition and compare it with that of halibut.

For this hydrolysis we have used the entire muscle substance, with the removal only of water-, alcohol-, and ether-soluble substances, as OSBORNE and HEYL employed. After separating the skin, bones, adherent fat and larger pieces of connective tissue, the remaining muscle was reduced to a pulp with a meat chopper, and extracted four times with several times its volume of water, then four times with absolute alcohol, finally with a large amount of ether until most of the fat was removed, then strained, pressed and dried.

I. Distribution of Nitrogen.

The muscle substance was hydrolyzed with concentrated hydrochloric acid, sp. gr. 1.19, in the usual way and the nitrogen was determined with the following results:—

	In per cent of air dry substance.	In per cent of dry matter.	In per cent of ash-and moisture- free substance.	Ratio. Total nitrogen as 100.
Total N	16.08	16.90	16.98	100.00
Hot hydrochloric acid soluble N	14.99	15.74	15.83	93.13

1. OSBORNE and HEYL: The Amer. Jour. of Physiol. XXIII, 81, 1908-1909.

2. WEISS: Zeitschr. f. physiol. Chem., 52, 108, 1907.

[Jour. Coll. Agric., Vol. V, No. 4, 1916.]

	In per cent of air dry substance.	In per cent of dry matter.	In per cent of ash-and moisture- free substance.	Ratio. Total nitrogen as 100.
In which: Ammonia N.....	1.03	1.08	1.09	6.39
Monoamino acid N	8.00	8.40	8.45	49.70
Organic base N	3.53	3.71	3.73	21.95
Other N	2.43	2.55	2.56	15.09
Hot hydrochloric acid insoluble N..	1.09	1.14	1.15	6.75
Ash	0.45	0.47	0.00	—

II. Mono-amino Acid.

389 gm. of the substance (moisture 4.85 per cent, ash 0.45 per cent) was hydrolyzed with three times its weight of concentrated hydrochloric acid, by boiling at first in water bath, then in oil bath, for about eight hours until biuret reaction had disappeared in the solution. The solution was evaporated into a syrup at low temperature under diminished pressure, and allowed to stand for a week in a cool place, after saturating with dry hydrochloric acid gas. 4.22 gm. of a crystal was obtained. It was not glutaminic acid hydrochloride, according to testing, but ammonium chloride, which sublimed at over 300°, gave brown coloration for NESSLER's solution and the following analytical results :

0.1412 gm. subst. gave	0.0371 gm. N
Calculated for NH_4Cl	26.18% N
Found	26.27% N

By concentrating and treating with absolute alcohol and dry hydrochloric acid gas, from the mother liquor together with washings, three crystals were obtained weighing respectively 2.79, 0.44, and 4.44 gm. These crystals were also ammonium chloride instead of glycocoll ethylester hydrochloride, having the following analytical data :

0.1426 gm. of the first crystal gave	0.0378 gm. N
0.3282 gm. of the second crystal gave	0.0846 gm. N
0.6564 gm. „ „ „ „ „	0.434 gm. Cl
Calculated for NH_4Cl	26.18% N 66.29% Cl
Found (the first cryst.)	26.46% N —

Found (the second cryst.) 25.80% N 66.12% Cl

The last crystal of ammonium chloride was somewhat impure, containing 24.5 per cent nitrogen. Some attempts were made to separate glutaminic acid, if present, and some crystals were isolated, but we could not identify them with certainty.

The mother liquor from the crystals of ammonium chloride was subjected to esterification in the usual manner, and after removing the ether, the esters were distilled with the following results:

Fraction.	Temperature.	Pressure.	Weight.
I.	till 60°	till 9 m.m.	26.5 gm.
II.	„ 80°	„ 7 „ „	18.9 „
III.	„ 100°	„ 4 „ „	70.0 „
IV.	„ 130°	„ 4 „ „	30.0 „
V.	„ 190°	„ 4 „ „	14.0 „
Total.....			159.4 „

The alcohol soluble portion of the fraction I, II and III.—Each fraction was saponified by boiling with ten times its volume of water, and the solutions were evaporated up to dryness, and extracted several times with absolute alcohol. The proline obtained from the alcoholic extract was converted into copper salt, which was separated through the treatment with absolute alcohol, into 2.24 gm. d, l-proline copper and 4.0 gm. l-proline copper. The racemic salt was dried at 120° and analyzed.

0.2050 gm. subst. gave	0.056 gm. Cu O
0.1426 gm. „ „	11.6 c.c. N (23 C°, 751.5 m.m.)
Calculated for C ₁₀ H ₁₆ O ₄ N ₂ Cu (proline copper)	21.79% Cu 9.60% N
Found	21.82% Cu 9.28% N

Alcohol insoluble portion of the fraction I, II and III.

Fraction I.—From 3.8 gm. of the mixture of amino-acids by treatment with absolute alcohol and dry hydrochloric acid gas, a needle crystal as glycocoll ethylester hydrochloride appeared, but it was very little, so we could not ascertain even the melting point with certainty. The mother solution was evaporated with water, boiled with lead oxide, filtered and converted into copper salt. The crystal thus obtained, weighing 1.2 gm., was crude alanine copper, containing 25.5 per cent copper (theoretical 26.5 per cent).

Fraction II.—10.5 gm. of crystals were separated at first into five fractions, and after examining the taste, melting point and the form of crystals in each, they were again united to two fractions, weighing respectively 4.0 and 6.5 gm. This second portion was united with the last 5 gm. crystals of Fraction III. 11.5 gm. of crystals, thus resulted, were converted into lead salt, by means of lead acetate and ammonia after LEVENE and VAN SLYKE,¹ and 4.5 gm. lead leucine was isolated. The filtrate of the crystals was decomposed with hydrogen sulphide, and the free amino-acids were fractionally crystallized, and then converted into copper salts by boiling with copper hydroxide freshly prepared. Thus we obtained 0.5 gm. of valine, and 3.0 gm. of alanine, which gave the following analysis :

0.1032 gm. subst. gave	0.0282 gm. CuO
0.2228 gm. „ „	0.0218 gm. N
Calc. for $C_{10}H_{20}N_2O_4Cu$ (valine copper)	21.33% Cu 9.47% N
Found	21.93% Cu 9.81% N
0.2854 gm. subst. gave	0.0932 gm. CuO
0.2590 gm. „ „	0.0307 gm. N
Calc. for $(C_3H_5NO_2)_2Cu$ (alanine copper)	26.52% Cu 11.69% N
Found	26.10% Cu 11.85% N

Fraction III.—45 gm. of amino-acids insoluble in alcohol was dissolved in water, and separated into twenty two portions by fractional crystallization, and in each the taste, the melting point and the form of crystals were observed and the nitrogen content was determined. The last 5 gm. was united and treated with Fraction II as above mentioned. From the remainder we get 26 gm. leucine, having the following composition :

0.1614 gm. of the first crystal gave	15 c.c. N (19° C, 761 m.m.)
0.4948 gm. „ „ fourth „ „	0.05464 gm. N
0.3121 gm. „ „ sixth „ „	0.03415 gm. N
0.1369 gm. „ „ sixth „ „	12.5 c.c. N (20° C, 758 m.m.)
0.8515 gm. „ „ twelfth „ „	0.0943 gm. N
0.1903 gm. „ „ tenth „ „	0.0476 gm. Cu O.
Calc. for $C_6H_{13}O_2N$ (leucine)	10.69% N

1. LEVENE and VAN SLYKE : Jour. Biol. Chem., 6, 391, 1903.

Found (the first cryst.)	10.88% N
„ („ fourth „)	10.92% N
„ („ sixth „)	10.91% N
„ („ „ „)	10.61% N
„ („ twelfth „)	11.09% N
Calc. for $(C_6H_{12}NO_2)_2Cu$ (leucine copper)	19.64% Cu
Found (the tenth cryst.)	19.98% Cu

The remaining crystals (ca. 14 gm.) were united with 4 gm. of the first crystals of Fraction II, and converted into lead salt. After the removal of 4 gm. leucine lead, the filtrate was decomposed by means of hydrogen sulphide, and fractionally crystallized. 1 gm. of valine was thus separated, but the larger portion of the crystals remained as a mixture. For the purpose of separating the valine from the mixture, we converted it into copper salt, and extracted it with methyl alcohol after EHRLICH and WENDEL.¹ 0.7 gm. valine copper was isolated, but the remainder was left without perfect separation. From the lead leucine, lead was removed and analyzed:

0.3601 gm. subst. gave	0.0369 gm. N
Calc. for $C_6H_{13}O_2N$ (leucine)	10.69% N
Found	10.24% N
0.2130 gm. subst. gave	0.0246 gm. N
Calc. for $C_5H_{11}O_2N$ (valine)	11.96% N
Found	11.55% N
0.1134 gm. subst. gave	0.0308 gm. Cu O
0.1987 gm. „ „	0.0184 gm. N
Calc. for $C_{10}H_{20}N_2O_4Cu$ (valine copper)	21.33% Cu 9.47% N
Found	21.52% Cu 9.28% N

Fraction IV and V.—These two fractions were united and treated with water. Phenylalanine ester, separated oily, was taken in ether and evaporated with hydrochloric acid. 21 gm. hydrochloride, thus obtained, melted at 225° and had the following analytical data:

0.2768 gm. subst. gave	15.8 c.c. N (21 C°, 764 m.m.)
0.1458 gm. „ „	0.0273 gm. Cl

1. EHRLICH and WENDEL: Biochem. Zeitschr. viii, 399, 1908.

Calc. for $C_9H_{11}O_2N \cdot HCl$ (phenylalanine hydrochloride)	6.95% N	17.58% Cl
Found	6.69% N	17.17% Cl

The aqueous solution, separated from the etherial layer, gave only 0.2 gm. barium aspartate, when it was saponified with baryta. After the removal of barium and saturating with dry hydrochloric acid gas, from the filtrate of the aspartate, we obtained 7.5 gm. glutaminic acid hydrochloride, which gave the following analytical result :

0.1453 gm. subst. gave	0.0283 gm. Cl
Calc. for $C_5H_{10}O_4NCl$ (glutaminic acid hydrochloride)	19.31% Cl
Found	19.45% Cl

From the mother liquor of the glutaminic acid hydrochloride, by completely removing the hydrochloric acid by boiling with lead oxide, and by precipitating the lead from the filtrate by means of hydrogen sulphide, 6.0 gm. aspartic acid which melted at 265° was obtained. This crystal was boiled with copper hydroxide to be converted into copper salt, but unfortunately, we lost it accidentally.

After neutralization and evaporation of the filtrate of aspartic acid, a little crystal was obtained, but we could not purify it.

III. Diamino-acids and Tyrosine.

42 gm. of the air-dried muscle was boiled with three times its weight of sulphuric acid and six times of water for fourteen hours. The crystal, which appeared on evaporation of the solution after the complete removal of sulphuric acid through baryta, was purified by boiling with acetic acid, and we obtained 1.05 gm. tyrosine, which melted at 303° , and gave the following analytical results :

0.1489 gm. subst. gave	0.0116 gm. N
Calc. for $C_9H_{11}O_3N$ (tyrosine)	7.74% N
Found	7.79% N

The filtrate of the tyrosine was examined according to Kossel's method, described by WEISS.¹ Arginine and lysine were isolated as picrate, and histidine as dichloride.

1. WEISS: Zeitschr. f. physiol. Chem, 52, 108, 1907.

The results of determination.

	In 100mg. air-dried substance.	In 100mg. ash and moisture. free substance.
	Nitrogen determined.	Base calculated. Base calculated.
Arginine	1.56	4.88 5.15
Histidine	0.53	1.96 2.07
Lysine	1.13	5.95 6.28

Arginine picrate. Yield 1.9 gm. M. P. 208°—210°.

0.5480 gm. subst. gave 0.3150 gm. picric acid

0.1960 gm. „ „ 0.0479 gm. N

Calc. for $C_6H_{14}N_4O_2 \cdot C_6H_3N_3O_7$ 56.81% picric acid 24.32% N

Found 57.48% picric acid 24.46% N

Lysine picrate. Yield 1.86 gm. M. P. 240°—242°.

0.3506 gm. subst. gave 0.2154 gm. picric acid

0.1020 gm. „ „ 0.0189 gm. N

Calc. for $C_6H_{14}N_2O_2 \cdot C_6H_3N_3O_7$ 61.07% picric acid 18.67% N

Found 61.44% picric acid 18.44% N

Histidine dichloride. Yield little. M. P. 235°.

It gave a red color by diazobenzol sulphonic acid and sodium hydroxide.

IV. Conclusion.

The results of hydrolysis of the material are given in the following table, and are compared with those obtained by OSBORNE and HEYL from the muscle substance of halibut.

	In per cent of ash- and moisture-free substance.	
	<i>Pagrus.</i>	Halibut.
Glycocoll.....	Absent or trace.	Absent.
Alanine	1.04	?
Valine	0.60	0.79
Valine containing leucine	2.17	—
Leucine	8.82	10.33
Proline	1.22	3.17
Phenylalanine	4.72	3.04
Aspartic acid	1.66	2.73

	In per cent of ash- and moisture-free substance.	
	<i>Pagrus.</i>	Halibut.
Glutaminic acid	1.63	10.13
Serine	?	?
Tyrosine	2.64	2.39
Arginine	5.15	6.34
Histidine	2.07	2.55
Lysine	6.28	7.45
Ammonia, determined	1.32	1.33
Ammonia, isolated	1.04	—
Tryptophane	Present	Present

From these figures, the amount of glycocoll and serine in fish muscles seems to be quite in harmony, being zero or trace in both cases.

The quantities of arginine, histidine and lysine in *Pagrus* are generally less than those in the halibut, but their ratio between the three bases is very similar in both muscles.

There is some disagreement in the proportion of proline, phenylalanine and asparatic acid between these two muscles.

Most distinct differences are found in the proportion of alanine and glutaminic acid. While over one percent of alanine was easily obtained from *Pagrus*, only a doubtful trace of this substance had been found from the halibut. The greatest quantitative difference was found for glutaminic acid, only one sixth as much being obtained from *Pagrus* as from the halibut. This may be partially attributed to the fact that we could not get very low pressure as in the case of the latter, but it must be noted that there is a most remarkable difference between the two, that in the direct separation of glutaminic acid from the hydrolyzed fluid before esterification, by evaporation and saturation of dry hydrochloric acid gas, we obtained from *Pagrus* much crystal of ammonium chloride, which was not the case with the halibut.

On the Nuclease of Fishes and Mollusca.

By

Yuzuru Okuda.

After the investigation of ARAKI,¹ who for the first time found the liquefaction of α -nucleic acid through the action of an enzyme, which was afterwards named nuclease by IWANOFF,² its occurrence in various animals and plants was ascertained, and some properties also explained in the meritorious works of PLENGE, SCHITTENHELM, NAKAYAMA, JONES, SHIGA, SACHS, ABDERHALDEN, KIKKOJI, DOX, MENDEL, WELLS, LEVENE, BLANCHARDIÈRE, and others.³ But it seems to me, that there is no work on this enzyme of fishes, therefore in the present work an attempt has been made to throw some light on the properties and the distribution of the enzyme in the tissues and organs of some fishes.

I. Distribution.

For this purpose, the extracts from the liver, spleen, hepatopaneas, ovary, testis, gill, pyloric coecum, mucosa of stomach and intestines were used.

The extracts were prepared from very fresh organs, after the removal of most of the adherent impurities, by washing the organs with abundant water, chopping, weighing, grinding up with some quartz sand to a paste, extracting with about three times its weight of chloroform water, and then

1. ARAKI: Zeitschr. f. physiol. Chem., 38, 84, 1903.

2. IWANOFF: Zeitschr. f. physiol. Chem., 39, 31, 1903.

3. PLENGE: Zeitschr. f. physiol. Chem., 39, 190, 1903.—SCHITTENHELM u. SCHRÖTER: *ibid.*, 39, 203, 1903.—SCHITTENHELM: *ibid.*, 42, 257, 1904.—NAKAYAMA: *ibid.*, 41, 348, 1904.—JONES: *ibid.*, 42, 35, 1904.—SHIGA: *ibid.*, 42, 502, 1904.—SACHS: *ibid.*, 46, 337, 1905.—ABDERHALDEN: *ibid.*, 47, 452, 1906.—KIKKOJI: *ibid.*, 51, 201, 1907.—DOX: J. of biol. Ch., 6, 461, 1909.—MENDEL & WELLS: Amer. J. of physiol., 24, 170, 1909.—WELLS: J. of biol. Ch., 7, 171, 1910.—LEVENE & MEDIGRECEANU: *ibid.*, 9, 65, 1911.—Blanchardière: Zeitschr. f. physiol. Ch., 87, 291, 1913.

pressing. Each 10 c.c. of the extract of an organ was put in two flasks, *A* and *B*, of which *B* was boiled. To both flasks, 10 c.c. of 3 per cent natrium nucleate solution, enough toluol and a little chloroform were added, stoppered, and kept for several days in an incubator, shaken from time to time. In every case aseptic precautions were observed as far as possible, and with the samples, in which no bacterial growth was observed, phosphoric acid and purin base liberated from nucleic acid was determined.

Determination of phosphoric acid.—After digestion, the action of enzyme was arrested by boiling the solution, then immediately filtered, if necessary acidifying with one drop of acetic acid. Phosphoric acid was determined in the filtrate by BÖTTCHER-WAGNER’S method.

Determination of purin base nitrogen.—Digested solutions were filtered, freed from protein and undecomposed nucleic acid by means of basic lead acetate, then freed from lead by sulphuric acid. Purin bases were precipitated from the filtrate after neutralization with sodium hydroxide, by the method of KRÜGER and SCHMID, and the nitrogen in the precipitate was determined by KJELDAHL’S method.

PAGRUS MAJOR.

Exp. 1. Extracts were prepared from the liver, pyloric coecum, mucosa of the stomach, and of the investines of five fresh fishes, by extracting with chloroform water for 15 hours at room temperature, and allowed to stand for 5 days at 28–31° C.

Extract.	Reaction.	Mg ₂ P ₂ O ₇ in gm.
Liver <i>A</i> ¹	Faintly acidic for litmus	0.043
„ <i>B</i> ²	—	Trace
Mucosa of stomach <i>A</i>	Amphichromatic	0.026
„ „ „ <i>B</i>	—	Trace
„ „ intestines . . <i>A</i>	Amphichromatic	0.036
„ „ „ . . <i>B</i>	—	0.005
Pyloric coecum <i>A</i>	Amphichromatic	0.015
„ „ <i>B</i>	—	Trace

1. *A*: Extract, 10 c.c., sodium nucleate, 3 per cent, 10 c.c., chloroform & toluol.
2. *B*: Extract, 10 c.c., boiled „ „ „ „ „

Exp. 2. Samples were obtained from four fresh fishes, by extracting the organs for 24 hours at room temperature, and incubating for 5 days at 32–33° C.

Extract.	Reaction.	Magnesium pyrophosphate in gm.	Purin base N in mgm.
Spleen <i>A</i>	Amphichromatic but rather acidic	0.042	24.0
„ <i>B</i>	—	0.009	9.5
Ovary ¹ <i>A</i>	Amphichromatic but rather basic	0.029	30.0
„ <i>B</i>	—	0.010	7.0
Flesh <i>A</i>	Faintly acidic	0.019	7.9
„ <i>B</i>	—	0.020	6.5
Blood	—	? ²	—
Bile	—	?	—

CYPRINUS CARPIO.

Samples were obtained from five living carps, by extracting the organs for 24 hours at room temperature, and keeping 5 days at 34–36° C.

Extract.	Reaction.	Magnesium pyro- phosphate in gm.
Mucosa of stomach ³ <i>A</i>	Amphichromatic	0.034
„ „ „ <i>B</i>	—	Trace
„ „ intestines .. <i>A</i>	Amphichromatic	0.030
„ „ „ <i>B</i>	—	Trace (?)
Spleen ⁴ <i>A</i>	Neutral	0.017
„ <i>B</i>	—	Trace
Gill <i>A</i>	Amphichromatic	0.033
„ <i>B</i>	—	0.006
Haepatopaneas <i>A</i>	Neutral	0.043
„ <i>B</i>	—	Little
Testis <i>A</i>	Neutral	0.030
„ <i>B</i>	—	0.005

1. Eggs were not yet matured.
2. Blood and bile: only trace of ammonium magnesium phosphate was obtained in *A* and *B*.
3. Stomach; the somewhat expanded part before the intestine was treated as the stomach.
4. Spleen; only 2 c.c. of the extract, instead of 10 c.c., was used.

Extract.	Reaction.	Magnesium pyro-phosphate in gm.
Flesh I..... <i>A</i>	Neutral	0.013
„ „..... <i>B</i>	—	0.012
„ II ¹ <i>A</i>	Acidic	0.016
„ „ <i>B</i>	„	0.013
Blood	—	?
Bile	—	?

KATSUWONUS PELAMYS.

Samples were obtained from two bonitos, by extracting the organs for 20 hours at room temperature, incubated for 5 days at 37–39° C.

Extract.	Reaction.	Magnesium pyrophosphate in gm.	Purin base N in mgm.
Pyloric coecum.... <i>A</i>	Amphichromatic	0.101	23.0
„ „..... <i>B</i>	—	0.021	5.3
Mucosa of stomach <i>A</i>	Amphichromatic rather acidic	0.037	13.1
„ „ „ <i>B</i>	—	Trace	2.2
„ „ intestines <i>A</i>	Amphichromatic	0.030	—
„ „ „ <i>B</i>	—	Trace	—
Liver..... <i>A</i>	Amphichromatic	0.074	16.2
„ <i>B</i>	—	0.010	1.8

SERIOLA QUINQUERADIATA.

Only flesh was examined.

Extract.	Reaction.	Magnesium pyro-phosphate in gm.
Flesh I..... <i>A</i>	Faintly acidic	0.038
„ „..... <i>B</i>	—	0.037
Flesh II <i>A</i>	Faintly acidic	0.041
„ „ <i>B</i>	—	0.036

MOLLUSCA.

The whole bodies of living mollusca were ground and extracted. The extracts, fresh and boiled, were allowed to stand for three days at 33–35° C. Phosphoric acid was then estimated only qualitatively.

1. Flesh II; both *A* and *B* were acidified with a few drops of acetic acid before incubation.

Extract.	Ppt. of ammonium magnesium phos- phate. ¹	Extract.	Ppt. of ammonium magnesium phos- phate.
<i>Corbicula laena</i> A	+++	<i>Arca inflata</i> A	+++
„ „ B	(+)	„ „ B	+
<i>Meretrix meretrix</i> A	+++	<i>Tapes philippinarum</i> A	++++
„ „ B	(+)	„ „ B	(+)

From the above experiments we see that the extracts from the liver, pyloric coecum, spleen, ovary, mucosa of the stomach and of the intestines of *Pagrus*; the hepatopancreas, spleen, testis, gill, mucosa of the stomach and of the intestines of the carp; the liver, pyloric coecum, mucosa of the stomach and of the intestines of the bonito, and organs of some mollusca contain active nuclease, liberating phosphoric acid and purin bases from yeast nucleic acid. But the existence of the enzyme seems to be only as trace in the examined flesh, and doubtful in blood and bile, on account of which the nucleic acid remained intact.

II. Isolation of Purin Bases.

For the purpose of isolating purin bases, liberated from nucleic acid decomposed by the enzyme, we made the following two experiments.

a. Liver.—To 40 c.c. of the liver extract from *Pagrus*, 120 c.c. of 3 per cent sodium nucleate solution, sufficient chloroform and toluol were added, and after digestion for five days at 28–31° C, the solution was filtered and freed from undecomposed nucleic acid by means of acetic acid and copper sulphate. From the filtrate, the excess of copper was removed by hydrogen sulphide, and purin bases were precipitated by sulphuric- and phosphotungstic acids. The precipitate was decomposed by baryta, and the baryta was removed by carbonic acid, and the filtrate was precipitated by nitric acid and silver nitrate solution. By ammonia, this was transformed into silver salt, which was decomposed by hydrochloric acid. From the filtrate of silver chloride, by concentrating and treating with ammonia, 0.04 gm. of crude guanine was obtained. It gave the diazobenzol sulphuric acid reaction and the xanthine-like reaction (nitric acid and alkali), and its picrate was a

1. (+), indicates trace; +, little; +++, much; +++, more.

yellow prismatic crystal, with difficulty soluble in water. From the ammoniacal filtrate, ammonia was removed, and adenine was isolated as picrate, which yielded 0.08 gm. and gave the following analytical results, after drying at 100° C in vacuum :

0.0462 gm. subst. gave	12.2 c.c. N	(17°, 478 m.m.)
Calculated for $C_5H_5N_5 \cdot C_6H_3N_3O_7$		30.79% N
Found		30.61% N

M. P.—280–282° C.

b. Digestive organs.—To 110 c.c. of the extract of the digestive organ₁ of *Pagrus*, 55 c.c. of 3 per cent sodium nucleate solution was added and treated as above mentioned. 0.02 gm. guanine and 0.128 gm adenine picrate thus resulted. The former gave the guanine reactions, and the latter the following results:

0.049 gm. subst. gave	13 c.c. N	(17°, 762.4 m.m.)
Calculated for $C_5H_5N_5 \cdot C_6H_3N_3O_7$		30.79% N
Found		31.30% N

M. P.—279–280° C.

III. Influence of Acid and Alkali.

Sulphuric acid, acetic acid, sodium carbonate, and sodium hydroxide were employed to ascertain the influence of OH or H ions, on the enzyme.

a. Sulphuric acid and sodium hydroxide. To the mixture of 10 c.c. of testis-extract of a carp and 10 c.c. of 3 per cent sodium nucleate solution, 5 c.c. of sulphuric acid or sodium hydroxide solution was added, and then the mixture was kept for 5 days at 34–36° C with sufficient toluol and chloroform.

Per cent of H_2SO_4 in solution.	$Mg_2P_2O_7$ in gm.	Per cent of $NaOH$ in solution.	$Mg_2P_2O_7$ in gm.
0.0230	0.043	0.028	0.012
0.0115	0.033	0.056	0.009
0.0023	0.034		

Control (no addition of acid or alkali)

Fresh extract 0.030 gm. $Mg_2P_2O_7$

Boiled extract 0.005 gm. $Hg_2P_2O_7$

1. The pyloric coecum, mucosa of stomach and intestines.

From these results it seems, that faint acid solution has a stimulating effect, while alkaline solution has a retarding influence upon the enzyme process. Nucleic acid, as we know, undergoes slight decomposition even at room temperature in an acid solution, but the decomposition of nucleic acid given above is not due simply to acid-action, but chiefly to the enzyme action, as the following experiment shows.

With the ovary-extract of *Pagrus*, experiments were repeated.

Extract, unboiled.	0.067% H_2SO_4	0.046 gm.	Mg. pyrophosphate.
Extract, boiled.	„ „	0.013 gm.	„ „
Extract, unboiled.	No addttion	0.029 gm.	„ „
Extract, boiled.	„ „	0.010 gm.	„ „

Even in the boiled extract, containing several times as much sulphuric acid as in former cases, the decomposition of nucleic acid can scarcely be recognized, while in the active extract the stimulating effect of the acid is again remarkable. Therefore, the results in the preceding experiments may be justified.

b. Acetic acid and sodium carbonate. To know the optimum concentration of acid for the enzyme action, 10 c.c. of liver extract of *Pagrus*, 5 c.c. of 3 per cent sodium nucleate solution, and 10 c.c. of acetic acid solution with different concentrations, were mixed and kept for 7 days at 31–33° C with antiseptics, and after that time phosphoric acid was determinationed.

Per cent of CH_3COOH in solution.	$\text{Mg}_2\text{P}_2\text{O}_7$ in gm.	Per cent of CH_3COOH in solution.	$\text{Mg}_2\text{P}_2\text{O}_7$ in gm.
0	0.042	0.20	0.043
0.02	0.042	0.30	0.035
0.05	0.049	0.30(boiled ext.)	0.006
0.10	0.046		

These results are also in harmony with those of sulphuric acid. And the optimum concentration of acetic acid for the action of enzyme of the liver of *Pagrus* seems to be 0.05–0.1 per cent.

In the next place, to know in what concentration of acid or alkali the enzyme action will be arrested, similar experiments were performed with the testis-extract of carps, kept for 5 days at 32–34° C.

Per cent of CH ₃ COOH in solution.	Mg ₂ P ₂ O ₇ in gm.	Per cent of Na ₂ CO ₃ in solution.	Mg ₂ P ₂ O ₇ in gm.
0.5	0.008	0.5	0.012
1.0	0.008	0 (boiled ext.)	0.006

The action seems to be completely arrested by 0.5 per cent acetic acid, and nearly so by 0.5 per cent sodium carbonate.

IV. Decomposition Temperature.

To kown the decomposition temperature of the enzyme, each 2 c.c. of the liver extract of *Pagrus* was taken in similar test tubes. After keeping for 5 minutes at the following temperature and immediately cooling in the test tubes, equal amounts of sodium nucleate and antiseptics were added, and allowed to stand for 4 days at 31–32° C, and then test for phosphoric acid was performed.

Temperature	50	60	70	80	90	100
NH ₄ Mg PO ₄ -ppt. ¹	+++	++	(+)	—	—	—

The result shows that the enzyme is destroyed between 70–80° C.

V. Optimum Temperature.

To known the optimum temperature of the enzyme, the mixture of 10 c.c. of the liver extract of *Pagrus*, 5 c.c. of sodium nucleate solution, 10 c.c. of water, a little chloroform and toluol was left for 4 days at the following temperatures. Then the flask containing the mixture was immersed in boiling water to arrest rapidly the enzyme action, filtered, and phosphoric acid was determined with the following results :

Temperature	5–10°	20–22°	24–25°	31–33°	32–35°
Mg ₂ P ₂ O ₇ in gm. ..	0.029	0.035	0.037	0.032	0.030

According to these results, the optimum temperature for the exzyme lies near 25°.

VI. Influence of Dilution of Solution.

To know whether the accumulation of the cleavage products of nucleic acid hinders the enzyme action or not, the following two experiments were

1. +, indicates presence ; —, absence ; (+), trace.

made, but as far as the experiments show, the influence could not be recognized.

Exp. 1. 7 c.c. of the extract of digestive organs of *Pagrus* 7 c.c. of sodium nucleate solution and antiseptics. 9 days at 31–34°.

Water added (c.c.).....	0	40	60
Total volume (c.c.) of the aqueous solution..	14	54	74
Magnesium pyrophosphate (gm.)	0.079	0.079	0.068

Exp. 2. 1 c.c. of the extract, pressed out from the mixture of the hepatopancreas and spleen of a carp. 10 c.c. of sodium nucleate solution, antiseptics. 6 days at 33–35°.

Water added (c.c.).....	0	10	100
Total volume (c.c.) of the aqueous solution..	11	21	111
Magnesium pyrophosphate (gm.).....	0.043	0.041	0.043

VII. Velocity of Decomposition.

To ascertain the velocity of the decomposition of nucleic acid by the enzyme, two experiments were made.

Exp. 1. A mixture of 40 c.c. of the liver extract of *Pagrus*, 40 c.c. of 3 per cent sodium nucleate solution, 40 c.c. of water, sufficient chloroform and toluol was prepared and kept at 31–33°. After 1, 3, 7, 9, 11, and 13 days respectively each 20 c.c. was taken for the determination of phosphoric acid, with the following results:

Days after preparation	1	3	7	9	11	13
Magnesium pyrophosphate (gm.)	0.024	0.030	0.032	0.023	0.029	0.035

Exp. 2. Two flasks, each containing a mixture of 10 c.c. of the liver extract of *Pagrus*, 10 c.c. of water, 5 c.c. of nucleate solution and some antiseptics, were left for 4 and 7 days respectively at 31–33° C, and then phosphoric acid was determined.

Days after preparation	4	7
Magnesium pyrophosphate (gm.)	0.032	0.042

The above results are somewhat irregular, but it seems that the decomposition goes on for about 4 days.

VIII. Isolation of the Enzyme.

For this purpose three experiments were made.

a.—About 100 gm. of the digestive organs of *Pagrus* was finely minced, ground with some quartz sand, macerated with 50 c.c. of water, and then strained through cloth. The extract was precipitated by saturation with zinc sulphate. The precipitate was treated with a little water and dialyzed in cold water. The enzyme action was examined, with positive results in the solution, with negative results in the boiled one.

b.—The liver extract of bonito. *c.*—The pyloric coecum extract of bonito. The extract was poured into a mixture of absolute alcohol and ether, the voluminous precipitate produced was collected on a filter, then washed with alcohol and ether. The crude enzyme thus prepared, when dissolved in a little water, showed distinct nuclease action, liberating phosphoric acid from nucleic acid, while control case remained always inactive. This method of isolation seems to be more convenient than the former.

IX. Summaries.

1. The existence of nuclease was ascertained in the liver, pyloric coecum, spleen, ovary, the mucosa of the stomach and of the intestines of *Pagrus*; in the hepatopaneas, spleen, testis, gill, in the mucosa of the stomach and of the intestines of the carp; in the liver, pyloric coecum, in the mucosa of the stomach and of the intestines of the bonito; and in the organs of some mollusca, but it was scarcely ascertained in the examined fish-flesh, blood and bile.

2. Guanine and adenine were obtained from yeast nucleic acid, decomposed by the action of the extracts of the liver, and digestive organs of *Pagrus*.

3. In all the examined cases, faintly acid solutions had a stimulating effect upon the enzyme action, while alkaline solutions a retarding influence. The optimum concentration of acid for the enzyme of the liver extract of *Pagrus* was 0.05—0.1 per cent acetic acid. The enzyme action of the carp's

testis was almost completely arrested by 0.5 per cent acetic acid, 0.5 per cent sodium carbonate, or 0.06 per cent sodium hydroxide.

4. The nuclease of *Pagrus* liver was completely destroyed by heating for five minutes under 80°, while according to SACHS¹ the enzyme of the pancreas of warm blooded animals remains still active by heating over that temperature.

5. The optimum temperature of the enzyme action of *Pagrus* liver lies near 25°, but even at as low a temperature as under 10° the action is remarkable.

6. The influence of the concentration of the cleavage products for the enzyme could not be recognized in the scope of the experiments performed.

7. The decomposition of nucleic acid through the enzyme goes on comparatively slowly even over 30°.

8. The enzyme was isolated by precipitating with zinc sulphate, or with a mixture of alcohol and ether.

1. SACHS: Zeitschr. f. physiol. Chem., 46, 337, 1905.

On the Existence of Inosinic Acid-splitting Enzyme in Fish-Organs and in *Aspergillus melleus*.

By

Yuzuru Okuda.

On the decomposition of inosinic acid by enzymatic action we have no literature except one treatise, which is furnished by the investigation of LEVENE and MEDIGRECEANU¹ with dog's organs. The writer has made here some experiments with fish-organs and *Aspergillus melleus*, which had been isolated by M. YUKAWA² from dried bonito, the so-called "Katsuobushi."

The inosinic acid employed in this work was isolated as barium salt, by the writer, from bonito-extract with HAISER-WENZEL'S method and HAISER'S method.³ For the experiment of enzyme the barium salt was converted into sodium salt by means of sodium sulphate.

I. Distribution of the Enzyme in Fish-Organs.

The organ extract prepared as described by the writer in the study of nuclease,⁴ was divided into two equal parts, and put into two flasks, one of which was immediately boiled. Both flasks were then shaken with some

1. LEVENE and MEDIGRECEANU: Jour. of biol. Chem., 9, 65, 1911.

2. YUKAWA: Jour. Coll. Agric. Tokyo, Vol. 1, 357, 1911.

3. HAISER-WENZEL: Abderhalden, Handb. d. biochem. Arbeitsmethoden, II, 602, 1910.--
HAISER: ibid, 599.

Isolated inosinic acid gave the following analytical results, when dried in vacuum at 100°.

0.336 gm. subst. gave 0.0726 gm. H₂ O.

Calc. for C₁₀ H₁₁ N₄ PO₈ Ba + H₂O + 6½ H₂ O—21.84% H₂ O: Found 21.60% H₂ O

0.177 gm. subst. gave 0.0854 gm. Ba SO₄.

Calc. for C₁₀ H₁₁ N₄ PO₈ Ba—28.42% Ba: Found 28.39% Ba.

But in some cases, somewhat impure inosinic acid, without further purification, was subjected to the experiment.

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sodium inosinate solution, enough toluol and a little chloroform, stoppered, and kept for a few days at a certain temperature. Aseptic precautions were observed as far as possible. After the time, the flasks were boiled and their contents were filtered. Phosphoric acid liberated from inosinic acid, or inosinic acid remaining undecomposed was then determined in the filtrate.

Phosphoric acid was directly determined by BÖTTCHER-WAGNER's method, as in the case of nuclease. Inosinic acid was precipitated by HAISER-WENZEL's method with basic lead acetate, and after the removal of the lead from the precipitate by means of hydrogen sulphide, the inosinic acid was transformed into phosphoric acid by ignition with magnesium nitrate, and the resulting phosphoric acid was determined by molybdic method.

Exp. 1. Extracts were obtained from several living carps. 10 c.c. of 1 per cent solution of inosinic acid was employed in each experiment. Each flask was kept for 3 days at 33°.

Extract.	Phosphoric acid as $\text{mg}_2 \text{P}_2 \text{O}_7$, in gm.	Inosinic acid as $\text{mg}_2 \text{P}_2 \text{O}_7$ in gm.
Mucosa of intestines, I, 5 c.c.	0.013	—
Do., boiled	Trace	—
Mucosa of intestines, II, 10 c.c.	0.028	Trace
Do., boiled	Trace	0.018
Flesh, I, 10 c.c.	0.029	—
Do., boiled	0.020	—
Flesh, II, 10 c.c.	0.023	—
Do., boiled	0.019	—
Flesh, II, 10 c.c. ¹	0.022	—
Do., boiled	0.018	—

With the flesh of the bonito and horse-mackerel, a similar experiment was performed with similar result as in the case of carp-flesh.

Exp. 2. Extract of carp's organ, 5 c.c. Inosinic acid solution, 3 per cent, 5 c.c. After keeping for 5 days at about 30°, phosphoric acid was precipitated as ammonium magnesium phosphate.

1. 10 c.c. of 1 per cent sodium nucleate solution, instead of inosinate solution, was used for the sake of comparison.

Extract.	Ppt. of NH_4MgPO_4 .	Extract.	Ppt. of NH_4MgPO_4 .
Hepatopaneas	+++	Do., boiled	(+)
Do., boiled	(+)	Gill	+++
Bile	—	Do., boiled	(+)
Do., boiled	—	Spleen	++++
Mucosa of stomach and intestines	++++	Do., boiled	(+)

From the experiments, we see that the above mentioned organ extracts of a carp have the power of decomposing inosinic acid with liberation of phosphoric acid, while the bile lacks the action. Disintegration of inosinic acid by the flesh examined is not remarkable but distinct.

II. Autolysis of Flesh.

To see the decomposition of inosinic acid present in flesh in course of autolysis, some experiments were performed. For the sake of convenience, hen-flesh was used for the purpose. A hen was bled to death and the flesh taken under aseptic precautions. The flesh was minced with a meat-chopping machine, and rubbed with some quartz sand in a mortar. 200 gm. of the paste thus prepared was divided into two equal parts and put in the flasks *A* and *B*. After adding 1000 c.c. of water to each flask, *B* was boiled for a few minutes to destroy the enzymatic action. Both flasks were then shaken with enough totuol and a little chloroform, and kept for 10 days at about 30°. After that time analysis was made with the following results:

	<i>A</i>	<i>B</i>
Phosphoric acid in 100 gm. of flesh. ¹	Unboiled	Boiled
Inorganic form (as $\text{Mg}_2\text{P}_2\text{O}_7$ in gm.)	0.516	0.474
Inosinic acid form („ „) (HAISER-WENZELS method)	0.009	0.086
Do. (HAISER's method)	0.004	0.075

From this it is evident, that inosinic acid is decomposed during the autolysis of the flesh. The same takes place in some other flesh. As inosinic acid has been found by S. KODAMA² to be an indispensable com-

1. Total phosphoric acid in 100 gm. of flesh corresponds to 0.49 gm. P_2O_5 or 0.768 gm. $\text{Mg}_2\text{P}_2\text{O}_7$.

2. KODAMA: Jour. Tokyo Chem. Soc., XXXIV, 751, 1913.

ponent of the taste of bonito flesh, its occurrence and the autolysis should have some meaning on some flesh as a food.

III. Existence of the Enzyme in *Aspergillus melleus*.

"Katsuobushi" is a Japanese food made of dried bonito by the action of some mould fungi. To know the relation between these microorganisms and inosinic acid, which seems to be a necessary constituent of the taste of the food, the following experiments have been made. As one of those microorganisms *Aspergillus melleus* was used.

Exp. 1. "Koji"-extract-culture of *Aspergillus melleus* was filtered and the filtrate was equally divided into two test tubes, one of which was boiled. Inosinic acid solution and antiseptics were then added to both. Increased amount of phosphoric acid in the unboiled test tube was ascertained after three days.

Exp. 2. Neutralized "koji"-extract containing two per cent sodium nucleate was divided equally into two ERLLENMEYER'S flasks, sterilized, and the microorganism was introduced. One of the two was immediately boiled, and after 5 days both were filtered and tested.

Sample.	Growth.	Phosphoric acid in the filtrate of the mould.	Phosphoric acid in the mould, after ignition.
Unboiled.	Abundant.	Trace.	Abundant.
Boiled.	No.	Trace.	—

Exp. 3. 31 gm. of the mould, cultured in "koji"-extract for 7 days, was separated from the culture solution, washed with water, ground up to a paste with some quartz sand and kieselguhr, treated with a little water, pressed out, and then filtered. With each 5 c.c. of the clear solution thus prepared experiments for the enzyme were made, adding 20 c.c. of 3 per cent inosinic acid solution, and antiseptics, and keeping for 5 days at room temperature. The unboiled sample gave 21 mg. $\text{Mg}_2\text{P}_2\text{O}_7$, while the boiled one only trace.

Exp. 4. A portion of the above enzyme solution was poured into a mixture of absolute alcohol and ether. The voluminous precipitate thus produced, when dissolved in a little water, has shown strong enzyme action.

From the above experiments, the existence of the enzyme in *Aspergillus melleus* is evident—an undesirable fact, assuming inosinic acid as an essential compound for “Katsuobushi.”¹ This enzyme is more or less diffused in culture solutions, and precipitated by alcohol and ether. *Aspergillus melleus* assimilates inosinic acid.

IV. Summary and Conclusion.

1. Enzymatic decomposition of inosinic acid was tested with positive results in the extracts of hepatopancreas, spleen, gill, the mucosa of the stomach and intestines of a carp, but with negative results in the bile. The distribution of the enzyme nearly harmonizes with that of the nuclease, investigated by the writer. These two enzymes will probably prove to be one and the same.

2. Inosinic acid in hen-flesh undergoes disintegration during the autolysis of the flesh. The same result will also be obtained in some fish containing inosinic acid.

3. *Aspergillus melleus* Yukawa decomposes inosinic acid. The enzyme, precipitated with alcohol-ether, is also powerful.

In conclusion, I wish to express my thanks to Mr. M. YUKAWA for his kindness in supplying the microorganism.

1. Unfortunately, I could not obtain any sample of *Aspergillus gymnosardae* Yukawa, which is a more common and important species for the preparation of “Katsuobushi.” It seems to me that it would be interesting to repeat the experiment with this species.

On the Relation between the Chemical Constituents of "Asakusa-nori" (*Porphyra laciniata*) and its Quality.

By

Hidesaburo Matsui.

(With the assistance of Y. Miyama)

"Asakusa-nori" is farmed on a large scale in the shallows of Tokio Bay. "Hoshi-nori" is made of "Asakusa-nori" in the following way: the raw material is gathered from the "Hibi,"¹ washed in fresh water, and chopped into small pieces. It is then distributed in water, made into thin sheets like paper, and dried in diffused light. This is a favorite food-stuff of the Japanese.

The quality of "Hoshi-nori" does not only depend upon its colour, lustre, taste and degree of softness, but also upon the art of manufacturing; the quality of the raw material (*i.e.* "Asakusa-nori") not always having anything to do with it.

Studies in the relation between the chemical constituents and quality of "Asakusa-nori" have been made by Mr. Y. OKUDA.² He concludes that the superior quality contains a larger amount of nitrogen than the inferior. The subject of my study is the same relation, and was made on selected materials of which the place of production was known. The experimental samples were of five different kinds, two produced at Urayasu,³ the others at Goi,³ in November. The results of my experiments are as follows:—

The data of the samples from Urayasu were as follows:—

1. "Hibi" is a bundle of brushwood stuck into the bottom of the sea, on which "Asakusa-nori" grows.
2. Journ. of Scien. Agric. Soc. 118.
3. Urayasu and Goi are villages on Tokio Bay, well-known as habitats of "Asakusa-nori."

	<i>A</i>	<i>B</i>	Amount of <i>B</i> for <i>A</i> as 100.
Market price (Sen) of one Jō (ten sheets)	9.0	3.0	
Average weight of one Jō.	22.75	22.75	
Market price for 100 grms.	40.48	13.49	
Moisture in 100 grms.	10.75	12.71	
Market price for 100 grms. of dried substance	44.33	15.10	34

Per cent in dried substance.

Ash	10.633	13.031	82 (reciprocal)
Chloride of sodium (calculated from the total soluble chlorine)	2.398	2.390	93 (reciprocal)
Fibre (by KÖNIG'S method)	20.915	28.295	74 (reciprocal)
Fat (ether extract)	2.145	1.516	71
Carbohydrate (calculated as glucose after hydrolysis)	29.729	20.643	69
Soluble matter	30.528	27.580	90
Total nitrogen	5.079	2.835	44
Albuminous do.	4.632	2.517	54
Soluble do.	1.337	2.699	52
Soluble albuminous do.	0.890	0.376	43
Non-albuminous do.	0.447	0.318	71
Organic bases do.	0.149	0.091	61
Mono-amino do.	0.044	0.007	16

From the above figures it will be seen that the superior quality (*A*) has more nitrogens, fat, carbohydrate and soluble matter, and less moisture, chloride and fibre than the inferior (*B*). This shows that the former was grown in a more favourable ground in the main current of the Yedo River, the water of which contains a large amount of nutrients for the algae, while the latter was grown more or less distant from the current. This is why the latter contains more fibre and chloride and less of the reserved substances than the former. The amount of nitrogens, especially soluble albuminous nitrogen (chiefly colouring matters) stands in close relation to the quality and market price, and carbohydrate, fat (lustre) and fibre (crispness) are next in importance.

The data of the Goi "Hoshi-nori" were as follows :—

	<i>A</i>	<i>B</i>	<i>C</i>
Market price per Jō (Sen)	3.2	2.2	2.0
Average weight do. (grm.)	21.5	22.0	22.0
Market price per 100 grms.	15.4	10.0	9.1
Moisture in 100 grms.	3.44	3.30	3.46
Market price per 100 grms. of dried subst.	15.9	10.3	9.6
Fibre in 100 grms.	32.401	32.526	35.943
Fat do.	1.243	1.200	1.077
Carbohydrate do.	23.971	22.980	23.220
Total nitrogen do.	3.281	3.276	3.251
Soluble nitrogen do.	1.491	1.371	0.746
Soluble albuminous nitrogen do.	1.193	0.894	0.452
Organic bases nitrogens do.	0.238	0.193	0.135

The three different kinds of Goi "Hoshi-nori" differ very little in price; the above-mentioned relations between the chemical constituents (nitrogens, especially the soluble albuminous form, fat, carbohydrate and fibre) and market prices may be clearly seen in *A*, *B* and *C*, the amount of carbohydrate not following the grading in the case of *C*.

CONCLUSION.

1. The quality of "Asakusa-nori" stands in intimate relation to the conditions of its growth and the nature of the ground; and the nitrogens, especially the soluble albuminous form (chiefly colouring matters), carbohydrate, fat and fibre have much influence on it.

2. The superior quality is obtained from a more suitable location, where the water is rich in nutritives for algae (for instance, fields along the main current of a river), and consequently contains larger amounts of all forms of nitrogens, carbohydrate, fat; and a less amount of fibre than the inferior.

3. Under the same conditions of manufacturing (place, season and handling), the market price of "Hoshi-nori" depends upon the quality of the material, *i.e.* "Asakusa-nori," so that goods of a higher price have more nutritive value as food-stuff than those of a lower price.

Studies in the Chemical Composition of "Tarabagani" (*Paralithodes Camtschatica*).

By

Hidesaburo Matsui.

The "Tarabagani" of Northern Japan has in recent years received considerable attention on account of its commercial value, as it is exported in large quantities to North America and Europe.

According to Mr. K. NAKAZAWA this species (*Paralithodes camtschatica*) is distributed in the Japan and Okhotsk Sea. It is caught in millions every year, especially on the west coast of Sakhalin Island and in the sea surrounding the Kurile Islands. It generally lives in depths from 100 to 200 fathoms, but in the breeding season, in spring, it comes up to a shallower sea of 50 fathoms. The full-grown male crab weighs about 8 kilos and its back shell measures 15 cm. in breadth. The female is about one-fifth of the male in weight. No studies of its chemical constituents and the differences between the male and the female have been attempted as far as I know and this induced me to take up this work, the result being as follows:—

THE FLESH.

This crab has a white fibrous flesh which tastes very nice and is therefore chiefly tinned for export. The figures shown in Table I show the differences between the chemical composition of the flesh in its raw state and of that once boiled in water.

The analytical samples were obtained on the west coast of Sakhalin Island in the month of May.

TABLE I.

	Per cent of original substance.		Per cent of dried substance.	
	Raw flesh.	Flesh once boiled.	Raw flesh.	Flesh once boiled.
Water	79.550	77.221		
Dry matter	20.450	22.779	100.00	100.00
Organic matter	18.993	12.411	92.58	94.00
Soluble do.	9.617	8.038	47.35	35.77
Protein	10.92	10.71	53.39	47.03
Inorganic matter	1.517	1.368	7.42	6.00
Soluble do.	1.432	1.097	7.00	4.82
Iron	0.0017	0.0012	0.0083	0.0053
Nitrogen	2.724	2.422	13.22	10.62
Albuminous do.	1.749	1.713	8.55	7.52
Non-albuminous do.	0.975	0.709	4.77	3.11
Insoluble do.	0.522	1.345	7.34	5.90
Soluble do.	1.202	1.077	5.88	4.72
Soluble albuminous do.	0.227	0.368	1.11	1.61
Organic bases do.	0.312	0.171	1.53	0.75

From the above figures it will be seen, that the dry matter in the flesh increases as the water decreases through boiling, and almost all the other contents also decrease with the water in which the flesh in boiled.

DIFFERENCES OF THE CHEMICAL COMPOSITION DUE TO SEX.

The samples for the experiments were canned at Kunashira-jima in the Kurile Islands in May, and a month later they were analysed with the following results :—

TABLE II.

	Per cent of original material.		Per cent of dried material.	
	The male.	The female.	The male.	The female.
Water	76.756	78.505		
Dry matter	23.244	21.495	100.00	100.00
Organic matter	21.093	19.218	90.75	89.414
Soluble do.	6.900	7.396	29.68	34.41

	Per cent of original material.		Per cent of dried material.	
	The male.	The female.	The male	The female.
Protein	16.332	17.78	17.41	82.73
Inorganic matter	2.151	2.277	9.25	10.59
Soluble do.	1.766	1.140	7.38	5.304
Iron	0.0030	0.00378	0.013	0.064
Sulphur	0.377	0.591	1.62	2.75
Nitrogen	3.038	2.841	13.07	13.21
Albuminous do.	2.613	2.205	11.25	10.25
Non-albuminous do.	0.425	0.636	1.82	2.96
Insoluble do.	2.216	1.969	9.534	6.20
Soluble do.	0.822	0.872	3.54	4.055
Soluble albuminous do.	0.397	0.236	1.71	1.10
Organic bases do.	0.043	0.099	0.185	0.66
Ammoniacal do.	0.033	0.027	0.12	0.125

According to the above figures, the flesh of the male crab contains less water, inorganic matter, iron, sulphur and non-albuminous nitrogen than that of the female.

THE BLOOD.

The blood clots the moment it has been taken from the living body, and forms a jelly like substance which turns dark blue in the air. On boiling it coagulates in an opaque white amorphous mass like the white of eggs. The blood, when mixed with a small quantity of tyrosine, at first changes into orange, then purple, dark blue, and finally into a black precipitate. Dilute alcoholic extract of the fresh blood free from coagulated albuminous masses gives weak reactions of oxydase by guajacol tincture, alkaline solution of α -naphthol and guaiacum tincture, but these colourations are distinctly given in the presence of hydrogen peroxide. Hydrogen peroxide is broken up by the fresh blood into H_2O and O .

SEXUAL DIFFERENCES OF BLOOD.

The blood of the male crab is almost colourless and that of the female slightly reddish. Their chemical compositions differ, as are shown in Table III. The materials for analysis were collected on the west coast of Sakhalin Island in spring.

TABLE III.

	Per cent of original material.		Per cent of dried material.	
	The male.	The female.	The male.	The female.
Water	94.941	95.081		
Dry matter	5.429	4.919	100.00	100.00
Organic matter	2.208	1.813	41.78	36.87
Protein	1.862	1.531	34.17	31.13
Inorganic matter	3.161	3.106	58.22	63.13
Sulphur	0.102	0.151	1.875	3.063
Nitrogen	0.329	0.291	6.045	5.97
Albuminous do.	0.298	0.245	5.47	4.98
Non-albuminous do.	0.031	0.046	0.58	0.99
Ammoniacal do.	0.020	0.011	0.22	0.73

These figures show that the dry matter in the blood is richer in the male than in the female, but the inorganic matter and sulphur in the dried substance is the reverse. The albuminous nitrogen is larger in quantity, but the non-albuminous is less in the male than in the female. The noticeable point of these figures is the presence of a considerable amount of sulphur in the dried blood of the female crab.

ASH OF BLOOD.

The analytical data of inorganic matter in the blood (♂) are as follows :—

TABLE IV.

	Per cent of the blood.			Per cent of total ash.
	Sol. in water.	Insol. in water.	Total.	
Total	2.6813	0.1869	2.8682	100.00
Na Cl	2.1578	nil.	2.1578	75.23
K Cl	0.3782	nil.	0.3782	13.18
Ca O	0.0350	0.0217	0.0567	1.98
Mg O				
SO ₃	0.0394	—	—	—
Si O ₂	nil.	0.0014	0.0014	0.049
Cu O	nil.	0.0079	0.0079	0.028
Fe ₂ O ₃	nil.	0.0050	0.0050	0.017
CO ₂				

From these figures we see that about nine-tenths of the ash in the blood composed of the chlorides of sodium and potassium, and the heavy metals such as iron and copper, are found in the blood as general constituents.

THE SHELL.

The shell of the living crab is of a dark purple colour, and it turns reddish when boiled with water or dipped in strong alcohol. Its chemical composition is shown in Table V.

TABLE V.

	Per cent of air-dried material.	Per cent of dried material.
Water	9.09	
Dry matter	90.91	100.00
Organic matter	68.08	74.89
Nitrogen	5.82	6.40
Carbonic acid as CO ₂	4.72	5.19
Crude ash	22.83	25.11
Ca O	10.49	11.54
P ₂ O ₅	4.02	4.42
SO ₃	1.26	1.39
Sand	0.34	0.38

Isolation and Identification of Glucosamine Hydrochloride :—The shell was first treated with dilute alkali and acid in order to remove the albuminous and mineral substances, after which it was washed with water and then completely decoloured by the action of K-permanganate. Finally it was decomposed with concentrated hydrochloric acid, and glucosamine hydrochloride was obtained in a crude state. It was recrystallized from dilute alcohol. The aqueous solution colours on boiling and reduces FEHLING'S solution. When boiled with caustic alkali solution it evolves ammoniacal vapour. The amounts of nitrogen (KJELDAHL'S method) and chlorine in it was as follows :—

0.2278 gm. of the subs. gave	0.0154 gm. of N.
Calc. for C ₆ H ₁₁ (NH ₂) O ₅ · HCl	: N=6.50%
Found	: N=6.76%
0.1032 gm. of the subs. gave	0.0168 gm. of Cl.

Calc. for $C_6H_{11}(NH_2)O_5 \cdot HCl$	: Cl=16.45%
Found	: Cl=16.29%

From these results it may be concluded that the crystals are glucosamine hydrochloride.

SUMMARY.

1. The dry matter in the flesh increase, on boiling and part of its soluble matter is lost at the same time.

2. The chemical composition of the flesh differs according to the sex, more water being found in the female than in the male, and also less of organic matter and protein in the dried flesh. Soluble matter, ash, iron, sulphur and nitrogen, the latter especially in non-albuminous form, are found less in the male.

3. The blood differs also according to the sex, the blood of the female has a larger amount of water, sulphur and non-albuminous nitrogen in the original substance.

4. The blood has oxydase, peroxydase, tyrosinase and catalase, therefore it colours dark blue on oxidation in the air. Heavy metals, such as iron and copper, are also general constituents.

5. Above one-fourth of the ash in the blood consists of sodium chloride.

6. The principal organic constituent of the shell is chitin, and the principal inorganic constituents are Ca-carbonate and Ca-phosphate.

On the Isolation of Putrescine by Steam Distillation of Putrefied "Agemaki" (*Solecurtus constricta*).

By

Hidesaburo Matsui.

Putrescine was first discovered by BRIEGAR¹ (1885) to be a ptomain, through his own method of investigating ptomaines with mercuric chloride, platinic chloride and similar reagents, and three years later UDRAUZKY and BAUMANN² (1888) proved the putrid base to be identical with tetramethylenediamine. Afterwards it was found by many investigators to exist in many putrid and autolysed substances, as will be seen from the following lists:—

UDRAUZKY and BAUMANN³ (1889): in some cases of cystinurea in urine.

CAUNNIDGE and GORROD⁴ (1900): Ibid.

LÖWY and NEUBERG⁵ (1904–5): Ibid.

GORROD and HURTLEY⁶ (1906): Ibid.

GARCIA⁷ (1892): in putrid horse meat.

ELLINGER⁸ (1900): by the action of putrefaction bacteria on ornithine.

DAKIN⁹ (1906): Ibid.

LAWROW¹⁰ (1901): in the autolysis of pig's stomach.

SCHENCK¹¹ (1905): in the autolysis of yeast.

1. Ptomaine 1885.
2. Berichte d. Deutsch. chem. Gesellschaft 21, 2938 [1888.]
3. Zeitschr. f. physiol. Chemie 13, 573 [1889.]
4. Journ. of Path. and Bact 6, 327–33 [1900.]
5. Zeitschr. f. physiol. Chemie 43, 355 [1904–05.]
6. Journ. of Physiol. 34, 217–23.
7. Ueber Ptomaine, welche bei der Fäulnis von Pferdefleisch und Pankreas entstehen I–IV.
8. Berichte d. Deutsch. chem. Gesellschaft 31, 3183 [1898.]
9. Journ. of biol. Chemistry 1, 171 [1906.]
10. Zeitschr. f. physiol. Chemie 33, 312 [1910.]
11. Wochenschr. f. Brauerei. 1905. No. 16.

KURONO¹ (1915) : Ibid.

WINTERSTEIN and THÖNY² (1902) : in Emmenthaler cheeses.

VAN SLYKE and HART³ (1902) : in Cheddar cheese.

RIELÄNDER⁴ (1908) : in ergot.

RENTER⁵ (1912) : in fresh specimens of *Boletus edulis*.

ACKERMANN⁶ (1909) : in the putrefaction products of protein.

YOSHIMURA⁷ (1910) : in putrid Soyabeans.

YOSHIMURA⁸ (1913) : in "Kazunoko," or dried roe of herring.

Isolation and Identification of Putrescine.—According to BARGAR⁹ putrescine is very little volatile with water, the author obtained it from a distillate which was prepared by passing steam into the alkaline solution of the putrid substance.

3 kilos of "Agemaki" with their shells were allowed to stand in 8 litres of water for 3 days. Putrefaction took place, the flesh was almost digested, the mass showed alkaline reaction and produced a disagreeable smell. It was acidified with hydrochloric acid and the suspending and albuminous bodies removed with tannic acid. The steam distillation was then carried out, at first in acidic and afterwards in alkaline reactions. Indol and skatol were not detected in the distillates. The volatile bases were slightly acidified with hydrochloric acid and evaporated to dryness on a water-bath. The residual mass was separated into a soluble (*A*) and an insoluble (*B*) portion by treating them with alcohol. The portion *B* consisted of white crystals. A small quantity of them was heated on a platinum plate, and evaporated after fusion without charring. The aqueous solution gave red precipitation by NESSLER's reagent, but nothing with mercuric chloride.

The portion *A* was a brownish coloured solution. It was concentrated to the consistency of syrup and allowed to stand over sulphuric acid in vacuum until white crystals were separated. The crystals were scarcely

1. Journ. of The Tokyo Chem. Soc. 36, 2 [1915.]

2. Zeitschr. f. physiol. Chemie 36, 28 [1902.]

3. New York Agric. Exper. Stat. 319, 204 [1902.]

4. Sitzungsber. der Gesellschaft z. Beförd. d. Naturw. Marburg 1908.

5. Zeitschr. f. physiol. Chemie 78, 167-245 [1912.]

6. Ibid. 60, 482 [1909.]

7. Biochem. Zeitschr. 28, 16 [1910.]

8. Journ. of the Tokyo Chem. Soc. 34, 3 [1913.]

9. The simple natural bases. 1914. [Monographs on Biochemistry.]

soluble in 96% alcohol. The insoluble crystals which did not precipitate with mercuric chloride were removed by treating them with 95% alcohol. The alcohol solution gave a white precipitate on the addition of a saturated alcohol solution of mercuric chloride. The precipitate was distributed in water, decomposed with hydrogen sulphide, and decolourized with animal charcoal. The decolourized solution was again evaporated to a small volume, and an aqueous saturated solution of sodium picrate was added to it. Glistening, yellow, thin platy crystals, which are hardly soluble in water and alcohol but insoluble in ether, were obtained after recrystallization being made from water. The product weighed about 0.43 gm. On being heated it begins to colour at about 230° C and melts at 250° C with decomposition. Picric acid was determined by the nitron method after being dried at 106° C.

Experiment 1. 0.1337 gm. of the picrate gave 0.2644 gm. of picric acid nitron; *i.e.*, 0.1129 gm. of picric acid.

Experiment 2. 0.0356 gm. of it gave 0.0708 gm. of picric acid nitron; *i.e.*, 0.0299 gm. of picric acid.

Calculated for $C_4H_{12}N_2 \cdot 2C_6H_2(NO_2)_3OH$: picric acid = 83.85%

Experiment 1. Found : „ „ = 83.70%

Experiment 2. Found : „ „ = 83.79%

Further, the picrate was decomposed with sulphuric acid, and the picric acid was then thoroughly extracted with ether. The platinic chloride was obtained by adding an aqueous solution of platinic chloride and evaporated to a small volume until crystals appeared on cooling. By this means, orange-yellow, prismatic crystals in aggregation were obtained. On being heated in a capillary tube, after recrystallization from water, and being dried at 106° C, it begins to colour at 208° C, turns black at 211°–213° C and decomposes at 227° C, without melting. The amount of platinum was determined with the following result:—

0.0233 gm. of the crystals gave 0.0093 gm. of platinum.

Calculated for $C_4H_{12}N_2 \cdot H_2PtCl_6$: Pt = 39.14%

Found : Pt = 39.00%

From these results, we may firmly conclude that the base isolated from the distillate of the putrid “Agemaki” was putrescine.

On the Chemical Changes of "Shiokara" during its Ripening and Preservation, and the Action of Common Salt on it.

By

Hidesaburo Matsui.

(With the assistance of Y. Miyama)

With two Text-Figures.

A few years ago Mr. OKUDA¹ published his investigation on the ripening of "Shiokara" made of certain organs² of bonito, concluding that the autolytic enzymes and the microbes that grow in the medium play an important rôle, and that soluble matter, non-albuminous nitrogen, especially monoamino nitrogen increase, and protein, organic bases, creatine, creatinine and purine bases decrease during the ripening process. The author's present report is on the "Shiokara" of cuttlefish.

EXPERIMENTAL PART.

The materials and preparation of the "Shiokara."

Experimental samples were made with fresh cuttlefish and a quantity of common salt (prepared by evaporation method with heat) at the Detached Marine Station of the Fishing Institute, Odawara.

Total weight of cuttlefish (67)	4.300	"Kwan" ³
Do. flesh	3.280	„
Do. livers	0.500	„

1. Original Communications, Eighth International Congress of Applied Chemistry. Vol. XVIII, 265.

2. The stomach, the intestines, and the pyloric cecum.

3. 1 "Kwan" = 3.7565 Kilos.

The following two samples (*A* and *B*) were made with the afore-said materials :—

	<i>A</i>	<i>B</i>
Flesh (minced)	1.520 "Kwan"	1.600 "Kwan"
Liver (ground)	0.190 "	0.200 "
Common salt	0.233 "	0.350 "
Do. %	13.55	19.44

Analytical data of the samples were as follows :—

	Per cent in original substance.		Per cent in dried substance.	
	<i>A</i>	<i>B</i>	<i>A</i>	<i>B</i>
Water	67.767	63.476	0.0	0.0
Dry matter	32.233	36.524	100.00	100.00
Organic matter	20.579	20.890	68.01	60.37
Crude protein	18.66	14.71	56.01	40.26
Total nitrogen	2.986	2.353	8.985	6.442
Ash	11.654	15.634	36.155	42.800
Chlorine as Na Cl	10.311	14.479	31.989	39.630

The mediums turned into alkaline reaction during the preservation. Many kinds of microbes were isolated from the "Shiokara."

The determinations of ammoniacal nitrogen were carried out with the original materials and total soluble, soluble albuminous, organic bases and monoamino nitrogens were estimated with the aqueous extracts.

The analysis was carried on for 104 days during the ripening and the preservation in winter (from November to March) with the following results :—

A

Per cent of original material.

Date.	Soluble N	Sol. alb. N	Nonalb. N	Org. bas. N	Monoamino N	Am. N
6th day	1.131	0.360	0.771	0.492	0.279	0.011
7	1.281	0.311	0.970	0.671	0.299	0.032
8	1.305	0.294	1.011	0.675	0.336	0.036
9	1.302	0.294	1.008	0.709	0.301	0.046
10	1.307	0.292	1.015	0.704	0.311	0.039
12	1.420	0.332	1.108	0.743	0.345	0.041
14	1.423	0.337	1.086	0.705	0.391	0.044
16	1.433	0.315	1.118	0.720	0.398	0.048
19	1.462	0.303	1.159	0.763	0.396	0.049
21	1.460	0.286	1.174	0.765	0.408	0.060
23	1.462	0.237	1.225	0.802	0.423	0.068
40	1.506	0.244	1.262	0.816	0.446	0.126
42	1.519	0.241	1.278	0.831	0.447	0.136
45	1.571	0.268	1.303	0.845	0.459	0.163
48	1.574	0.259	1.315	0.840	0.476	0.171
51	1.587	0.250	1.337	0.856	0.481	0.173
54	1.605	0.226	1.379	0.886	0.491	0.203
57	1.615	0.216	1.399	0.896	0.502	0.227
61	1.625	0.208	1.417	0.904	0.523	0.225
65	1.693	0.205	1.488	0.931	0.557	0.243
69	1.729	0.203	1.526	0.896	0.604	0.250
73	1.752	0.202	1.550	0.874	0.672	0.261
77	1.806	0.201	1.605	0.848	0.757	0.274
84	1.913	0.307	1.606	0.706	0.900	0.287
91	1.954	0.251	1.703	0.793	0.910	0.291
97	1.984	0.230	1.754	0.833	0.921	0.315
104	1.991	0.213	1.778	0.785	0.993	0.329

B

Per cent of original material.

Date.	Soluble N	Sol. alb. N	Nonalb. N	Org. bas. N	Monoamino N	Am. N
6th day	1.144	0.295	0.849	0.672	0.176	0.006
7	1.208	0.326	0.882	0.695	0.188	0.023
8	1.244	0.320	0.924	0.726	0.199	0.025
9	1.284	0.310	0.974	0.763	0.210	0.026
10	1.288	0.321	0.967	0.758	0.210	0.027
12	1.314	0.319	0.995	0.765	0.231	0.029
14	1.296	0.306	0.990	0.754	0.236	0.031
16	1.306	0.315	0.991	0.750	0.240	0.031
17	1.317	0.315	1.002	0.746	0.256	0.047
21	1.320	0.315	1.005	0.745	0.256	0.047
23	1.320	0.310	1.010	0.746	0.261	0.056
40	1.372	0.323	1.049	0.732	0.317	0.058
42	1.375	0.313	1.062	0.743	0.318	0.058
45	1.378	0.292	1.086	0.760	0.326	0.060
48	1.386	0.282	1.104	0.777	0.327	0.060
51	1.421	0.299	1.122	0.764	0.358	0.060
54	1.474	0.292	1.182	0.794	0.388	0.062
57	1.488	0.290	1.198	0.801	0.397	0.063
61	1.497	0.339	1.158	0.751	0.408	0.065
65	1.509	0.323	1.186	0.748	0.438	0.065
69	1.523	0.312	1.211	0.733	0.478	0.067
73	1.553	0.302	1.251	0.758	0.493	0.067
77	1.593	0.292	1.301	0.769	0.532	0.072
84	1.615	0.370	1.345	0.677	0.567	0.079
91	1.736	0.341	1.395	0.700	0.691	0.086
97	1.825	0.320	1.505	0.743	0.762	0.086
104	1.850	0.313	1.537	0.752	0.785	0.087

The above mentioned results may be diagrammatically represented as follows (Figs. 1 and 2):—

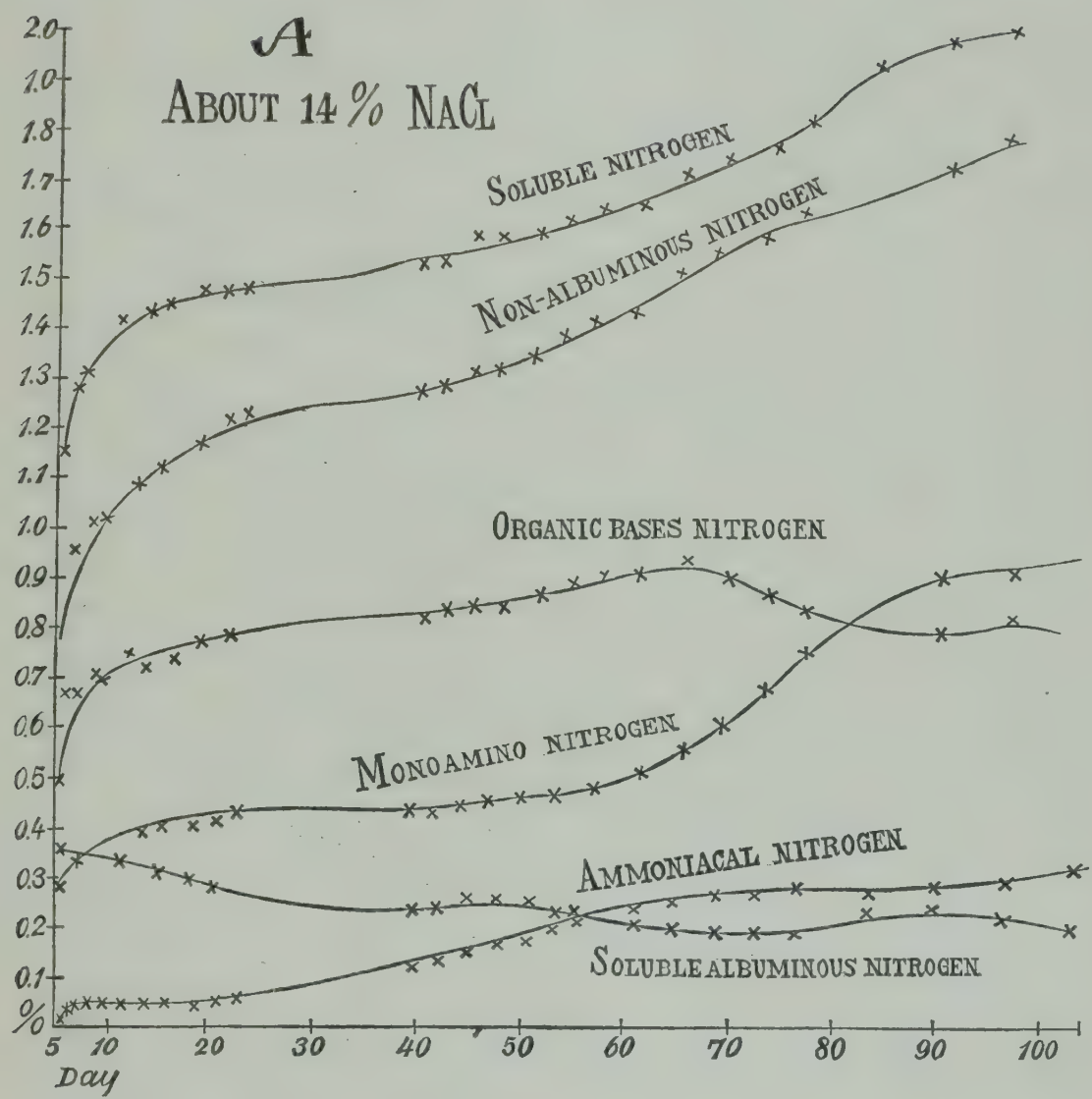


Fig. 1.

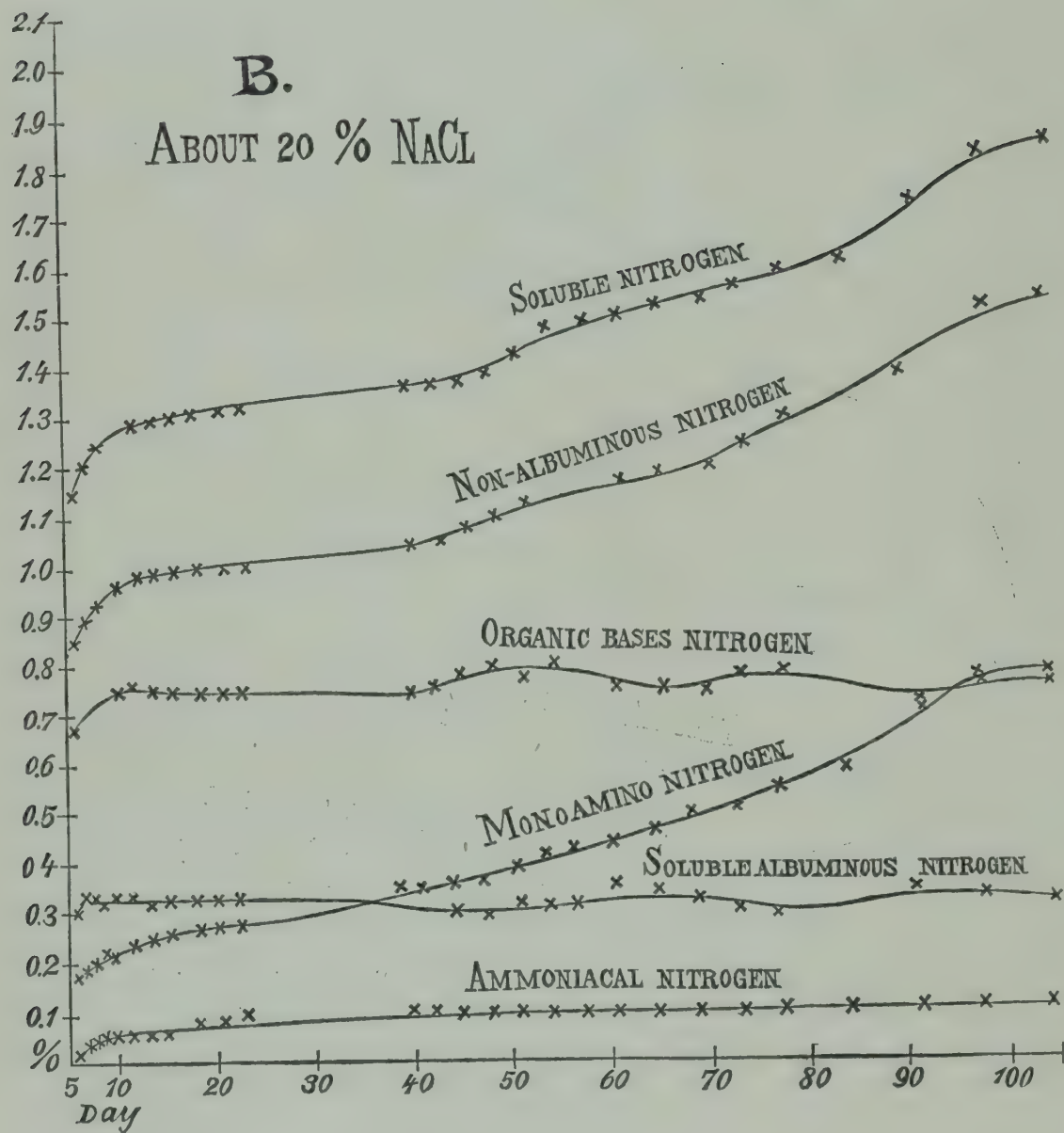


Fig. 2

SUMMARY.

1. During the ripening and the preservation of "Shiokara" of cuttlefish, insoluble nitrogenous compounds of the tissues and organs were decomposed into soluble forms, and the amount of nitrogen in the aqueous extract, therefore, gradually increased.

2. The decomposition rapidly advanced in the early period, then it gradually decreased for some time, but later it increased again.

3. The decomposition in the early period may be due to the enzymation of enzymes in the liver and tissues, and in the later period to the action of microbes.

4. The principal chemical changes during the ripening stage are the increase of organic bases (involved peptones), and those during the preservation are determined by the increase of monoamino acids and ammonia. The alkalinity of "Shiokara" during the preservation is due to the production of ammonia in the free state.

5. The action of common salt on "Shiokara" presents itself when the latter has been long in preservation. The production of amino-acids, especially ammonia, is lessened by the addition of 15% of common salt.

Chemical Studies in Some Marine Algae, Chief Material of “Kanten.”

By

Hidesaburo Matsui.

“Kanten,” or Japanese agar-agar, which is made from certain kinds of marine algae, is exported from this country all over the world. It is consumed as a food-stuff and for other purposes.

There are many processes of manufacturing “Kanten,” most of which have been patented. The oldest and commonest method is as follows:—The raw material is boiled with a proper quantity of water in an iron kettle for several hours, the digested portion is then separated from the fibrous residue by filtering through linen while it is warm. The filtrate solidifies on cooling, this is called “Tokoroten.” The solidified mass is then exposed to the cold night air (-5 to -15°C), so that the water freezes in it in crystals. The next day when the ice melts, the soluble substances, both organic and inorganic, in the “Tokoroten” are for the most part removed with the water. By repeating the above-mentioned process the substance is purified, until colourless “Kanten,” which is insoluble in water, is prepared.

“Tengusa” (*Gelidium*) is the best material and is principally used for the manufacture of “Kanten,” “Yegonori” (*Camphylaphora Hypnaeoides*) and “Ogonori” (*Gracilaria*) are used as accessories in the manufacture. These algae are decolourized before being used.

Qualitative determination: The three decolourized materials give the following results:—

	“Tengusa.”	“Yegonori.”	“Ogonori.”
FEHLING’S test.	—	—	—
Do. after hydrolysis with dilute acid.	str. reduced	str. reduced	str. reduced

	"Tengusa."	"Yegonori."	"Ogonori."
Orcin test.	yellow	yellow	yellow
BIAL's orcin test. ¹	green	green	green
SCHIFF's aniline acetate test for furfurol.*	cherry red	cherry red	cherry red
Phloroglucine test.*	+	+	+
TOLLENS and OSHIMA's test for methyl-pentosan.* ²	+	+	+
Resorcine test. ³	red	red	red
PINOFF's test for ketose. ⁴	+	+(strong)	+(weak)
I—KI—test.	reddish orange	reddish purple	reddish orange
Starch.	—	—	—
Mannite.	—	—	—
Formation of mucic acid by oxidation.	+	+	+

According to the above results, it may be concluded that the three de-colourized algae contain hexosans—aldose (galactan) and ketose—, pentosan and methyl pentosan, but not starch, mannite, nor reducing-sugars. Iod reaction on "Yegonori" and PINOFF's test on "Ogonori" are both noticeable.

Quantitative determination: Ash, nitrogen (KJELDAHL's method), fibre, galactan, pentosan, methylpentosan, and reducing-sugars after being hydrolized with acid in the de-colourized materials were determined by the following methods:—The dry matter was respectively in "Tengusa," 96.26%; in "Yegonori," 83.54%, and in "Ogonori," 97.10%.

Fibre:—For estimation of the marine algae fibre KÖNIG's glycerin-sulphuric acid method was used, because it is preferable to the method of HENNEBERG and STOHMANN. The substance was digested with his reagent in the autoclave at 140° C for an hour, the non-digested residue was then separated from the brownish mother liquor by the use of a tared GOOCH crucible with a sucking pump, and the residue was thoroughly washed off with hot water, alcohol, and finally with ether, and weighed after drying. The weight of the ash was then subtracted from it.

* With the distillate which is prepared by distilling the substance with 12% HCl.

1. Biochem. Zeitschrift, 3, 323.

2. Berichte d. deutsch. chem. Gesellschaft, 34, 1425.

3. Seliwanoff's resorcin test. Ibid., 20, 181.

4. PINOFF's test for ketose: Ketose colour green when boiled with both phosphomolybdic acid solution and acetic acid.

	"Tengusa."	"Yegonori."	"Ogonori."
Fibre (gram.) in 3 grms. of substance.	0.5170	0.3071	0.1262
Percentage in air-dry substance.	17.23	10.24	4.21
Percentage in dry matter.	17.89	12.25	4.32

Galactan:—The method of determining galactan as mucic acid was discovered by TOLLENS and his co-workers. The conversion of galactan into mucic acid is carried out by oxidation with nitric acid, 100 parts of this acid are theoretically obtainable from 116.66 parts of galactose, but in practice about 75% of galactose yields as mucic acid. The weight of galactan, therefore, may be calculated by multiplying the weight of mucic acid by $4/3 \times 9/10$. The author improved this method as follows:—The substance was first digested with 1% HNO_3 on a water-bath and the fibrous residue removed by filtration. Thus the extracted sugars were oxydized with strong nitric acid.

	"Tengusa."	"Yegonori."	"Ogonori."
Mucic acid (grm.) separated from 5 grms. of substance (average of several determinations.)	0.9335	0.8684	0.8979
Percentage in air-dry substance.	18.67	17.37	17.96
Percentage in dry matter.	19.80	20.78	18.50
Do. calculated as galactan.	23.70	24.88	22.14

Pentosan and methylpentosan:—The quantitative determination of these were studied by TOLLENS,^{1,2,3} KRÖBER,¹ ELLET² and MAYER.³ The method is based on their conversion into furfural and methylfurfural respectively, by distilling the substance with 12% hydrochloric acid (sp. gr. 1.06), and both furfurols are precipitated by means of a precipitant. Phloroglucin was used for this purpose in this analysis. At first both phloroglucides were precipitated at the same time and the former was separated from the latter by boiling the mixture in strong alcohol, because the latter is soluble in alcohol but not the former.

1. TOLLENS and KRÖBER: Jour. f. Landwirtsch. (1900), 355, (1901), 7.
2. TOLLENS and ELLET: Berichts d. deutsch. chem. Gesellschaft, 38, 492.
3. TOLLENS and MAYER: Zeits. d. Vereins d. deutsch. Zuck. (1907), 620; Berichte d. deutsch. chem. Gesellsch. 40, 2441.

	Samples.	Total phlorog.	Furfurol phlorog.	Methyl furf. phlorog.	Calculated as pentosan	Calculated as methyl pent.
"Tengusa" (grms.)	1.5799	0.03997	0.03433	0.00563	0.03504	0.01422
Per cent in dry matter	—	—	2.257	0.3701	2.303	0.9348
"Yegonori" (grms.)	1.6715	0.0899	0.07615	0.01325	0.07246	0.02961
Per cent in dry matter	—	—	5.454	1.185	5.189	2.125
"Ogonori" (grms.)	1.9419	0.0298	0.0205	0.0093	0.02278	0.01821
Per cent in dry matter	—	—	1.087	0.4933	1.209	0.966

N. B. The above-shown data are the average of two or three determinations.

The formulae for calculating pentosan and methyl-pentosan are as follows:—

$$\text{Pentosan} = (\text{Ph} + 0.0052) \times 0.8866$$

$$\text{Methylpentosan} = 1.85 \text{ MPh} - 6.25 \text{ MPh}^2 + 0.0040$$

where Ph and MPh represent respectively the amounts of phloroglucides of furfurol and methylfurfurol.

Reducing-sugars: The substance was hydrolized with 2% sulphuric acid for 10 hours by direct flame, and colouring or nitrogenous matters were precipitated by adding phosphotungstic acid. Reducing-sugars in the filtrate of the phosphotungstic precipitate after removal of the phosphotungstic and sulphuric acids with baryta in the usual way, were determined by PAVY-KUMAGAWA and SUTŌ's method. Reducing-sugars were calculated as glucose.

The above data when summarized and compared with the other investigators' data come out as follows:—

	Per cent of dried material.				
	"Tengusa." KÖNIG.		"Yegonori."	"Ogonori." KAWAKAMI.	
Ash	4.23	6.16	3.04	3.54	6.71
Lime	0.23	—	0.48	0.39	—
Magnesia	0.52	—	0.94	0.84	—
Alumina	—	—	0.45	0.55	—

	Per cent of dried material.				
	“Tengusa” KÖNIG.		“Yegonori.”	“Ogonori.” KAWAKAMI.	
Nitrogen	2.01	2.97	2.19	0.69	1.445
Crude protein (N × 6.25)	12.56	18.58	13.67	4.29	9.03
Fiber	17.89	14.71	12.25	4.32	—
Galactan	23.70	—	24.88	22.14	22.70
Pentosan	2.30	3.66	5.19	1.21	} 1.94
Methyl pentosan	0.93	1.21	2.13	0.97	
Reducing sugars after hydroliza- tion with dilute acid	23.20*	—	48.38	45.20	—

* Hydrolysis was carried out in a boiling water-bath instead of by direct flame

On New Protamines.

By

Makoto Yamagawa.

With ten Text-figures.

I. Introduction.

In 1874, MIESCHER (1) isolated a basic compound from the sperm of Rhine-salmon and called it "protamines", and in the same year PICCARD (3) published his research on this substance. 20 years later, in 1894, KOSSEL (2) isolated a similar compound, "sturine," from sturgeon sperm and then "salmine" and "clupeine" from salmon and herring sperm respectively, and he proposed to employ the term "protamine" as the group name for this kind of compounds.

KOSSEL and his pupils isolated further a series of protamines from different kinds of fish, namely: "arbasine" from sea-urchin sperm (by MATHEW), "scombrine" from mackerel sperm (by KURAJEFF) (5), "cyclopterin" from *seehasen sperm* (by MORKOWIN) (6), "cyprinine" from carp sperm (by KOSSEL and DAKIN) (7), "accipenserine" from *Accipenser stellatus* sperm (by KURAJEFF) (8), and "limandine" from flounder (*karei*) sperm (by GOTO) (2) etc. Some of these protamines were believed to be identical with others, so that they are not all different compounds. For instance, KOSSEL and KURAJEFF (4) believed clupeine to be identical with MIESCHER's salmine. This, however, was disproved by GOTO (9), who analyzed all the protamines then known, and found that slight but distinct differences exist between them. Of the above named protamines, arbasine has been found to contain sulphur, so that it should be excluded from the group "protamines," as the latter term is conventionally given to those compounds not containing sulphur.

MIESCHER (1) precipitated the protamine by platinic chloride, after

partially neutralizing the fluid which was obtained by treating salmon-sperm with dilute hydrochloric acid, and thus the double salt of protamine platinic chloride was obtained. As KOSSEL (2) failed to verify MIESCHER's result from salmon, he employed fat-free sperm of sturgeon. Moreover, he treated the sperm with 1% sulphuric acid, and caused precipitation of a protamine by adding three times its volume of alcohol. KURAJEFF, MORKOWIN, GOTO, and others obtained the protamines by KOSSEL's method. KURAJEFF (5) stated that the extracted fluid from sperm was of light red colour, but this may have been caused by being kept in sulphuric acid and exposed too long to light, or the material not having been completely washed. They purified the sulphate thus isolated, first by converting it into picrate and then re-changing it into sulphate. They further dissolved the sulphate in hot water. As the solution cools, an oily mass settles at the bottom, and after dissolving this in water the protamine sulphate was precipitated by alcohol. For analysis, they generally used the double salt of platinic chloride, sulphate, and chromate, but KURAJEFF (5) seems to think chromate the best form for elementary analysis.

Though the molecular weights of the protamines are not yet determined, their elementary compositions are given as follows:—

TABLE I. (10)

	Origin.	C%	H%	N%	Pt%	Cl%	O%	S%
Salmine (11) (platinic-chloride)	Salmon.	22.96	4.32	14.83	24.73	26.56	6.7	
Clupeine (11) (platinic-chloride)	Herring.	22.81	4.30	12.56	24.64	26.57	9.09	
Scombrine (11) platinic-chloride)	Mackerel.	23.94	4.75	13.57	24.09	25.99	8.11	
Sturine (11) (platinic-chloride)	Sturgeon.	24.32	4.49	14.20	23.10	25.42	8.47	
Cyclopteryne (sulphate)	<i>Cyclopterus lumpus.</i>	42.00	6.73	22.37				8.10
Cyprinine (sulphate)	Carp.							
Silurine	<i>Silurus glanis.</i>							
Accipenserine (13) (sulphate)	<i>Accipenser stellatus.</i>	32.83	6.25	19.41				10.02
Limandine (13) (platinic-chloride)	<i>Limanda yokohamae.</i>	30.75	5.13	13.29	18.49	20.17	12.15	

Protamine is a white, amorphous, very hygroscopic powder, insoluble in alcohol and ether, but soluble in water, and its aqueous solution is strongly alkaline and has laevorotatory power for polarized light. In aqueous solution, it does not coagulate through heat. By cooling a concentrated hot aqueous solution of the sulphate, a soluble oily mass is separated from the rest. The aqueous solution of protamine gives intense biuret reaction as well as histidine reaction by diazobenzol-sulphonic acid, but HOPKIN-COLE'S reaction is negative, as is also MILLON'S reaction, except in the case of cyprinine which, however, may be due to the presence of a little propepton or pepton, as MORKOWIN stated. The protamine is precipitated by alkaloidal reagents. WITTE'S pepton also causes precipitation in the presence of ammonia. Its aqueous solution is salted out by sodium chloride and ammonium sulphate, and KURAJEFF (5) tried to isolate it in crystalline form by utilizing this property, but without any success. The protamine is rich in nitrogen, but sulphur is absent, thus differing from histone. And though pepsin (12) does not act upon protamine, trypsin (12) and erepsin decompose it into crystalline cleavage products.

THOMPSON (13) investigated the physiological actions of protamine on dogs and reported as follows:—It is so poisonous that the dog was killed by 15–18 mgr. of salmine, clupeine and scombrine, and by 20–25 mgr. of sturine per kilogram of the live-weight; smaller quantities depressed the blood-pressure, decreased the number of white corpuscles in the circulation system and delayed the coagulation of the blood. But protone, the intermediate product, had a less poisonous property, and the final products, as hexon-bases, had no similar properties. As above described, protamine produces on hydrolysis sometimes a small quantity of monoamino acids but the chief products only consist of hexon-bases, in which from 50% to 80% is usually arginine. Table II represents the cleavage products of the protamines.

TABLE II (14).

	Salmine.	Sturine.	Clupeine.	Scombrine.	Cyclopterine.	Cypri- nine 1.	Cypri- nine 2.
Glycine.	—	—	—	—	—	—	—
Alanine.	—	—	—	—	—	—	—

	Salmine.	Sturine.	Clupeine.	Scombrine.	Cyclop- terine.	Cypri- nine 1.	Cypri- nine 2.
Valine.	4.3	—	—	—	—	—	—
Leucine.	—	—	—	—	—	—	—
Isoleucine.	—	—	—	—	—	—	—
Phenylalanine.	—	—	—	—	—	—	—
Tyrosine.	—	—	—	—	8.3	?	—
Serine.	7.8	—	—	—	—	—	—
Cystine.	—	—	—	—	—	—	—
Proline.	11.0	—	—	—	—	—	—
Oxyproline.	—	—	—	—	—	—	—
Aspartic acid.	—	—	—	—	—	—	—
Glutaminic acid	—	—	—	—	—	—	—
Tryptophane.	—	—	—	—	—	—	—
Arginine.	87.4	58.2	82.2	—	62.5	4.9	—
Lysine.	0	12.0	0	0	—	28.8	—
Histidine.	0	12.9	0	0	—	0	0
Diaminotriox- y-dodecanic acid.	—	—	—	—	—	—	—
Ammonia.	—	—	—	—	—	—	—
Total.	110.5	83.1	82.2	—	70.8	33.7	—

As the Japanese people, from early times, did not practise much cattle raising, the necessary nitrogen-food has been obtained from the sea, and marine products have been of important consideration. Hence fishing, and curing, have been considerably developed, and not only fish-flesh is used as food, but some of the inner organs in the form of "Shiokara," and "Karasumi" etc. Fresh roe is eaten boiled or else as "Kazunoko" and "Sujiko," but the milt of some fish is not eaten, because in many cases it tastes bad or is poisonous. In spite of the fact that fish-flesh is such an important food for our people, its chemical properties have not been much investigated, and as for spawn, scarcely any literature exists. The following is a small contribution to this interesting department of physiological chemistry, and it is hoped that further researches may be undertaken to shed more light on the very obscure question of Piscatorial chemistry.

II. Chemical Properties of the New Protamines.

In the present research six kinds of fish were used, namely, "suzuki," "nibe," "mutsu," "buri," "sawara," and "ishinagi."

The method of preparation is the same in all six cases and may be described as follows:—

The preparation of the protamine-sulphate: The ripe sperm of the fish was carefully washed with distilled water to remove blood and foreign matter, and then crushed by a meat-chopper. It was put in a bottle with a fairly large quantity of distilled water, and shaken continuously for one hour. This muddy mass was filtered through cloth under hand-pressure into a tall beaker, a few drops of acetic acid were added, then it was stirred and the precipitation of the suspended matter followed. If the upper liquid is not transparent, more acetic acid should be added to make it transparent, as otherwise the precipitation is incomplete and filtration is difficult. After letting it stand for several days, the upper part was sucked off and the residue boiled with ethyl alcohol. The boiling with ethyl alcohol and filtering was repeated several times; the residue was shaken with ether. These processes are necessary to remove the fatty substance, the absence of which may be inferred from the disappearance of colouration. The residue was dried on filter-paper at room-temperature. The substance thus prepared was dry fat-free sperm, and its percentage in fresh matter was as follows:—

TABLE III.

	Name of fish.	Dry fat-free sperm.
1.	Suzuki.	8.0%
2.	Nibe.	6.5%
3.	Mutsu.	6.7%
4.	Buri.	6.7%
5.	Sawara.	9.6%
6.	Ishinagi.	5.1%

A weighed quantity of the dry fat-free sperm was put into a bottle with a large quantity of 1% sulphuric acid solution, shaken continuously for one

hour on a shaking-machine, then sucked off, and the residue treated repeatedly in the same way until the sulphuric acid extract caused no precipitate with about three times the volume of absolute alcohol. The extracts were united, a large volume of absolute alcohol was added, and the vessel put in a dark place for twenty-four hours. (The precipitate by absolute alcohol is generally coloured by sunlight or too slow manipulation.) Then filtered, washed with ethyl alcohol, and finally with ether, and kept in a sulphuric-acid-vacuum-desiccator in a dark place.

The purification of the sulphate: At first I dissolved the isolated precipitate in a large quantity of distilled water, and heated; and after several minutes I filtered, neutralized with ammonia, and precipitated the free protamine by absolute alcohol. After two days the precipitate was collected, dissolved again in a large volume of water, and precipitated with an excess of saturated solution of sodium picrate. The yellow picrate thus prepared was filtered, ground with distilled water in a mortar, and sucked off with the "nutsche." This manipulation must be repeated to remove the impurities. After washing, the picrate was suspended in water, and acidified with sulphuric acid, to convert the picrate into sulphate. Picric acid set free was then removed by shaking the mixture with ether, which was repeated several times, until the ether-extract showed no colour. From this sulphuric acid-solution, protamine sulphate was precipitated by adding a large volume of absolute alcohol, after decantation it was filtered, washed with absolute alcohol and ether, and dried in a sulphuric-acid-vacuum-desiccator.

The preparation of the double salt of platinum chloride:—To prepare the double salt I dissolved a weighed quantity of the sulphate in distilled water, heated it on the water bath, and added sufficient barium chloride solution to remove the sulphuric acid, boiled it again, cooled and filtered. The filtrate was evaporated to a small volume and after adding a small quantity of concentrated hydrochloric acid so as to change it into chloride, I dissolved it in a large quantity of methyl alcohol. By adding a small excess of platinic-chloride-methyl alcohol solution, I obtained an amorphous, orange-red or light brown precipitate which was sucked off, washed with methyl alcohol and ether, and dried in sulphuric-acid-vacuum-desiccator.

1. Protamine from the sperm of "suzuki" [*Lateolabrax japonicus* (C. et V.) Tanaka]: *Lateoline*.

The material from fish caught in January was ripe and poor in fat.

50 gr. of dry fat-free sperm was shaken with 300 c.c. of 1% sulphuric acid for one hour, and this process was repeated 5 times. The protamine sulphate was precipitated by absolute alcohol and purified as above mentioned.

The average quantity of pure sulphate obtained in three cases was about 9% of dry fat-free sperm.

TABLE IV.

	Dry fat-free sperm.	Sulphate.
1.	50 gr.	4.3 gr.
2.	50 gr.	4.5 gr.
3.	100 gr.	9.2 gr.

Properties of the sulphate:—This sulphate is a hygroscopic, white, amorphous powder, whose base is strongly alkaline and uncoagulable in heat in the state of aqueous solution, and very soluble in water, but insoluble in alcohol and ether. It gives strong biuret reaction, but neither MILLON'S nor HOPKIN-COLE'S reaction. It also shows strong red colouration with diazobenzol-sulphonic acid, and is precipitated by phospho-wolframic acid, phospho-molybdic acid, mercuric chloride, potassium-iodine-iodide, potassium ferro cyanide, and potassium chromate solution, but not by silver nitrate. It is salted out by sodium chloride and ammonium sulphate from the solution. For the determination of the specific rotatory power, 0.2312 gr. of this sulphate was dissolved in 25 c.c. distilled water and observed through a tube 20 cm. long:

Observed angle of rotation : $= - 1.75^\circ$

Specific rotatory power : $[\alpha]_{\text{D}}^{20} = -94.61$

The platinum-chloride-double salt:—The double salt prepared by the above method is an orange-red powder, insoluble in warm water, methyl alcohol and ether. It was dried at 105° C. and analysed with the following results :

TABLE V.

	Subst.	Gave.
1	0.1162 gr.	0.1292 gr. CO ₂ and 0.0584 gr. H ₂ O
2	0.1156 gr.	0.1284 gr. CO ₂ and 0.0582 gr. H ₂ O
3	0.1168 gr.	18.8 c.c. N (20.0° C. 765.6 mm.)
4	0.1193 gr.	18.5 c.c. N (12° C. 765 mm.)
5	0.1176 gr.	0.0192 gr. Pt
6	0.1158 gr.	0.0638 gr. Ag=0.0110 gr. Cl.
7	0.1176 gr.	0.0646 gr. Ag=0.0111 gr. Cl.

TABLE VI.

	I.	Observed II.	Average.	Calculated for C ₃₀ H ₆₅ N ₁₆ O ₈ ·2HCl+PtCl ₄ .
C	30.32	30.29	30.31	30.33%
H	5.58	5.59	5.59	5.64%
N	18.92	18.88	18.90	18.87%
Pt	16.33	—	16.33	16.44%
Cl	18.11	18.06	18.09	17.93%

As this compound is analogous to the known protamines in origin, properties, preparation and other points, we conclude that it is a new protamine, and shall call it "lateoline" from the zoological name of the fish from which this substance was obtained, as A. KOSSEL did in the case of protamines isolated by him.

2. Protamine from the sperm of "nibe" [*Sciaena schlegelii* (Bleeker) Tanaka]: *Sciaenine*.

The material was the ripe milt of a fish caught in February, and fairly rich in fat. It was kept in cold storage at about -10° C. for 2 days and treated as described above. The prepared dry fat-free sperm was extracted just as in the case of "suzuki," the process being repeated five times. The solution of the sulphate was of slightly red-brown colour, but by repeating the purification this colour disappeared. The yield of the sulphate was as follows :

TABLE VII.

	Dry fat-free sperm.	Sulphate.
1.	50 gr.	3.8 gr.
2.	50 gr.	3.8 gr.
3.	100 gr.	7.8 gr.
4.	100 gr.	7.7 gr.

Properties of the sulphate:—The sulphate is a white amorphous powder, whose base is strongly alkaline and uncoagulable in heat in the state of an aqueous solution, and insoluble in alcohol and ether, slightly soluble in cold water but easily in hot water. Its aqueous solution gives weak biuret reaction, but no MILLON'S reaction. It gives strong diazobenzolsulphonic acid reaction when it is in slightly alkaline state; Hopkin-Cole's reaction is nearly negative. It is precipitated from the aqueous solution by phospho-wolframic acid, phospho-molybdic acid, mercuric chloride, potassium-iodine-iodide, potassium ferrocyanide and potassium chromate solution, but not by silver nitrate. It is salted out like lateoline by sodium chloride and ammonium sulphate. Dissolving 0.1090 gr. of this sulphate in 25 c.c. of distilled water, and determining the rotatory power of the polarized light (Na) through a tube 20 cm. long, the following result was obtained:

Observed angle of rotation: $= -0.56^\circ$

Specific rotatory power: $[\alpha]_D^{20} = -128.44$

The platinum-chloride-double salt:—The prepared double salt is a light brown powder, insoluble in water, methyl alcohol and ether. It is free from sulphur. The analysis of the dried (at 105°C.) sample is as follows:—

TABLE VIII.

	Subst.	Gave.
1	0.1166 gr.	0.0912 gr. CO_2 and 0.0617 gr. H_2O
2	0.1160 gr.	0.0908 gr. CO_2 and 0.0613 gr. H_2O
3	0.1251 gr.	17.7 c.c. N (17.5°C. 753.5 mm.)
4	0.1134 gr.	15.5 c.c. N (12.0°C. 760 mm.)
5	0.1174 gr.	0.0226 gr. Pt
6	0.1158 gr.	0.0762 gr. Ag=0.0250 gr. Cl.
7	0.1168 gr.	0.0750 gr. Ag=0.0246 gr. Cl.

TABLE IX.

	I.	Observed II.	Average.	Calculated for $C_{18}H_{58}N_{12}O_{10}2HCl \cdot PtCl_4$
C	21.33	21.35	21.34	21.34
H	5.88	5.87	5.88	5.93
N	16.60	16.61	16.61	16.60
Pt	19.25	—	19.25	19.29
Cl	21.07	21.11	21.09	21.03

As this compound is analogous to the known protamines in origin etc., we presume it to be a new protamine and shall call it “sciaenine,” as in the case of “lateoline.”

3. Protamines from the sperm of “mutsu” [*Scombrops boops* (Houtt.) Tanaka]: *Scombropine*.

The material were ripe milts of fishes caught in March, and fatter than those of “nibe.” They were kept in 80% alcohol for 24 hours. The milts were repeatedly washed with distilled water to free them from alcohol and treated as described in the above two cases, except that we used 100 gr. of sample, and 500 c.c. of 1% sulphuric acid, and that we repeated the shaking seven times instead of five.

TABLE X.

The average yield of the sulphate in four cases is about 7.5%.

	Dry fat-free sperm.	Sulphate.
1.	100 gr.	7.4 gr.
2.	100 gr.	7.5 gr.
3.	100 gr.	7.5 gr.
4.	100 gr.	7.6 gr.

Properties of the sulphate:—The salt is a white, amorphous powder, whose base is strongly alkaline and uncoagulable in heat in the state of an aqueous solution, and insoluble in alcohol and ether, easily soluble in water, the aqueous solution being colourless. The aqueous solution of the sulphate

gives intense biuret and diazobenzol-sulphonic acid reaction, but neither MILLON's nor HOPKIN-COLE's reaction. It is precipitated by phospho-wolframic acid, phospho-molybdic acid, mercuric chloride, potassium-iodine-iodide, potassium ferrocyanide, potassium chromate and silver nitrate, and it is also salted out by sodium chloride and ammonium sulphate. Dissolving 0.2316 gr. of this sulphate in 25 c.c. of distilled water and determining the rotatory power of the polarized light (Na) through a tube 20 cm. long, the following result was obtained:

Observed angle of rotation: $= -1.42^\circ$

Specific rotatory power: $[\alpha]_D^{20} = -76.64$

The platinum chloride double salt:—The prepared salt is a light brown powder, insoluble in water, methyl alcohol and ether. Sulphur is absent. It was dried at 105°C . and analysed:

TABLE XI.

	Subst.	Gave.
1	0.1162 gr.	0.1280 gr. CO_2 and 0.0620 gr. H_2O
2	0.1154 gr.	0.1270 gr. CO_2 and 0.0614 gr. H_2O
3	0.1140 gr.	15.6 c.c. N (11°C 767 mm.)
4	0.1144 gr.	15.6 c.c. N (10°C 767 mm.)
5	0.1404 gr.	0.0274 gr. Pt
6	0.1166 gr.	0.0754 gr. Ag=0.0248 gr. Cl.
7	0.1194 gr.	0.0770 gr. Ag=0.0253 gr. Cl.

TABLE XII.

	Observed.		Average.	Calculated for $\text{C}_{25}\text{H}_{57}\text{N}_{12}\text{O}_4 \cdot 2\text{HCl} \cdot \text{PtCl}_4$
	I.	II.		
C	30.04	30.01	30.03	30.03
H	5.93	5.91	5.92	5.91
N	16.87	16.88	16.83	16.82
Pt	19.52	—	19.52	19.54
Cl	21.26	21.22	21.24	21.30

Considering origin etc., we presume it to be a new protamine and shall call it "scombropine."

4. Protamine from the sperm of "buri" (*Seriola aureovittata* T. et S.): *Serioline*.

The material from fishes caught in March were ripe and nearly ripe milts, relatively rich in fat. As many blood vessels were running through the inner parts of the milts, they could not be well separated from the latter. The dry fat-free sperm, which was prepared by the method already described, was of light brown colour. The method used to prepare the sulphate was similar to that used above, but 600 c.c. of 1% sulphuric acid per 100 gr. of the sample was used each time, and the extraction was repeated eight times. By purification, however, we obtained a white powder which gives a colourless aqueous solution.

TABLE XIII.

The average yield of the sulphate in four cases.

	Dry fat-free sperm.	Sulphate.
1.	50 gr.	3.1 gr.
2.	50 gr.	3.1 gr.
3.	100 gr.	6.4 gr.
4.	100 gr.	6.5 gr.

Properties of the sulphate:—This salt is a hygroscopic, white amorphous powder, whose base is strongly alkaline and uncoagulable in heat in the state of an aqueous solution, and insoluble in alcohol and ether, but soluble in cold water. The aqueous solution of this salt gives intense biuret and diazobenzol-sulphonic acid reactions, but no MILLON'S and HOPKIN-COLE'S reactions. From the aqueous solution, it is precipitated by phospho-wolframic acid, phospho-molybdic acid, mercuric chloride, potassium iodine-iodide, potassium ferrocyanide and potassium chromate, but it is not precipitated by silver nitrate. It is also salted out from the aqueous solution by sodium chloride and ammonium sulphate. Dissolving 0.2120 gr. of this sulphate in 25 c.c. distilled water and determining the rotatory power of the polarized light (Na) through a tube 20 cm. long, the following result was obtained:

Observed angle of rotation : $= -1.50^\circ$

Specific rotatory power : $[\alpha]_D^{20} = -88.44$

Properties of the platinum chloride double salt:—It is a yellowish brown powder insoluble in methyl alcohol, ether and hot water. It is found to be free from sulphur. It was dried at 105°C and analysed:

TABLE XIV.

	Subst.	Gave.
1	0.1178 gr.	0.1438 gr. CO_2 and 0.0616 gr. H_2O
2	0.1172 gr.	0.1430 gr. CO_2 and 0.0610 gr. H_2O
3	0.1178 gr.	17.2 c.c. N (18°C . 763 mm.)
4	0.1140 gr.	16.2 c.c. N (10.5°C . 759 mm.)
5	0.1146 gr.	0.0213 gr. Pt
6	0.1144 gr.	0.0709 gr. Ag=0.0232 gr. Cl.
7	0.1146 gr.	0.0709 gr. Ag=0.0232 gr. Cl.

TABLE XV.

	Observed.		Average.	Calculated for $\text{C}_{29}\text{H}_{59}\text{N}_{13}\text{O}_3 \cdot 2\text{HCl} \cdot \text{PtCl}_4$
	I.	II.		
C	33.29	33.28	33.28	33.24
H	5.81	5.78	5.80	5.83
N	17.31	17.36	17.34	17.38
Pt	18.59	—	18.59	18.64
Cl	20.37	20.34	20.36	20.32

This compound is thus found to be a new protamine, and we shall call it "serioline."

5. Protamine from the sperm of "sawara" (*Scombreomorus nipponius*, C. et V.): *Scombremine*.

The material from fishes caught in April was nearly ripe, and so poor in fat that it could be made fat-free by simply boiling twice with alcohol.

The preparation of the sulphate was similar to the method mentioned above; 100 gr. of the sample were shaken with 500 c.c. of 1% sulphuric acid and the extraction was repeated five times. Even after repeated purification

the aqueous solution of the sulphate was not quite colourless. The average yield of the sulphate was as follows:—

TABLE XVI.

	Dry fat-free sperm.	Sulphate.
1	100 gr.	7.8 gr.
2	100 gr.	7.8 gr.
3	100 gr.	7.8 gr.

Properties of the sulphate:—It is a hygroscopic, white amorphous powder, whose base is strongly alkaline and uncoagulable in heat in the state of an aqueous solution, and insoluble in alcohol and ether, but very easily soluble in water. Biuret and diazobenzol-sulphonic acid reactions are intense; MILLON'S reaction is slightly positive, although all known protamines except cycloptertine have no MILLON'S reaction, and there is no HOPKIN-COLE'S reaction. It is precipitated by phospho-wolframic acid, phospho-molybdic acid, mercuric chloride, potassium-iodine-iodide, potassium ferrocyanide and potassium chromate, but there is no precipitation by silver nitrate from the aqueous solution. It is salted out by sodium chloride and ammonium sulphate. Dissolving 0.2240 gr. of this sulphate in distilled water into 25 c.c. and determining the rotatory power of the polarized light (Na) through a tube 20 cm. long, the following result was obtained:

Observed angle of rotation : $= -2.12^\circ$

Specific rotatory power : $[\alpha]_D^{20} = -118.30$

The platinum chloride double salt:—It is an amorphous, reddish-orange powder insoluble in methyl alcohol, ether and hot water. Sulphur is absent. It was dried at 105°C and analysed.

TABLE XVII.

	Subst.	Gave.
1	0.1080 gr.	0.1228 gr. CO_2 and 0.0574 gr. H_2O
2	0.1158 gr.	0.1318 gr. CO_2 and 0.0616 gr. H_2O
3	0.1180 gr.	16.7 c.c. N (18.5°C . 764.5 mm.)

	Subst.	Gave.
4	0.1185 gr.	16.7 c.c. N (18.5° C. 765.0 mm.)
5	0.1150 gr.	0.0206 gr. Pt
6	0.1150 gr.	0.0688 gr. Ag=0.0226 gr. Cl.
7	0.1148 gr.	0.0684 gr. Ag=0.0225 gr. Cl.

TABLE XVIII.

	Observed.		Average.	Calculated for $C_{28}H_{62}N_{13}C_6 \cdot 2HCl \cdot PtCl_4$
	I.	II.		
C	31.01	31.04	31.03	30.94
H	5.91	5.91	5.91	5.89
N	16.66	16.72	16.69	16.76
Pt	17.91	—	17.91	17.97
Cl	19.66	19.64	19.65	19.59

Thus we find the substance to be a new protamine, which we shall call “scombremine.”

6. Protamine from the sperm of “ishinagi” [*Stereolepis ishinagi*, (Helgd) Tanaka]: *Stereoline*.

The material was the ripe milt of a fish which was caught in May, and kept in ice-storage at $-10^{\circ}C$. for two weeks.

The yield of the sulphate was as follows:—

TABLE XIX.

	Dry fat-free sperm.	Sulphate.
1	100 gr.	11.2 gr.
2	100 gr.	11.0 gr.
3	100 gr.	11.0 gr.

Properties of the sulphate:—It is a hygroscopic, amorphous, white powder, whose base is strongly alkaline and uncoagulable in heat in the state of an aqueous solution, and insoluble in alcohol and ether, but easily soluble in water, the solution being colourless and clear. It gives strong biuret reaction but MILLON’S and HOPKIN-COLE’S reactions are negative.

Diazobenzol-sulphonic acid gives positive reaction, though it is weaker than in the cases of the above mentioned five sulphates. It is precipitated by phospho-wolframic acid, phosphomolybdic acid, mercuric chloride, potassium iodine-iodide, potassium ferrocyanide and potassium chromate; it is not precipitated by silver nitrate, the solution being of light red colour, and by sodium chloride and ammonium sulphate it is salted out from the aqueous solution. Dissolving 0.2252 gr. of this sulphate in 25 c.c. distilled water and determining the rotatory power of the polarized light (Na) through a tube 20 cm. long, the following result was obtained :

Observed angle of rotation : $= -1.75^\circ$

Specific rotatory power : $[\alpha]_D^{20} = -97.14$

The platinum chloride double salt :—It is a light reddish yellow powder containing no sulphur. It is insoluble in methyl alcohol, ether and even in hot water.

TABLE XX.

	Subst.	Gave.
1	0.1164 gr.	0.1206 gr. CO ₂ and 0.0642 gr. H ₂ O
2	0.1166 gr.	0.1208 gr. CO ₂ and 0.0645 gr. H ₂ O
3	0.1166 gr.	17.7 c.c. N (13.5° C. 765 mm.)
4	0.1168 gr.	17.7 c.c. N (13° C. 765 mm.)
5	0.1178 gr.	0.0216 gr. Pt.
6	0.1186 gr.	0.0722 gr. Ag=0.0237 gr. Cl.
7	0.1156 gr.	0.0704 gr. Ag=0.0231 gr. Cl.

TABLE XXI.

	Observed.		Average.	Calculated for C ₂₅ H ₆₄ N ₁₄ O ₆ ·2HCl·PtCl ₄ ·
	I.	II.		
C	28.26	28.26	28.26	28.14
H	6.13	6.15	6.14	6.19
N	18.42	18.43	18.43	18.39
Pt	18.34	—	18.34	18.31
Cl	20.01	20.02	20.02	19.96

Thus we conclude that the compound is a new protamine, and shall call it "stereoline."

HYDROLYSIS OF THE PROTAMINES.

2–3 gr. of the protamine sulphate, dried at 105° C, was boiled with 30 c.c. of 20% sulphuric acid in a flask, connected with reflux cooler for 8–10 hours. After cooling it was filtered.

The insoluble residue (melanin) was directly subjected to the nitrogen determination after KJELDAHL'S method. The filtrate was filled up to a certain volume from which a portion served directly for the determination of total nitrogen. A portion was precipitated with phospho-wolframic acid, the precipitate was washed with 5% sulphuric acid and subjected to nitrogen determination (total organic bases and ammonia-nitrogen). The filtrate of the phospho-wolframic acid precipitate was divided into two portions, one being used directly for KJELDAHL'S method, while the other portion, after removing the phospho-wolframic acid by baryta, was used for the determination of amino-nitrogen by the nitrous acid method after VAN SLYKE.

Finally the ammoniacal nitrogen was determined by neutralizing the sulphuric acid with caustic lime and subjecting it to vacuum distillation.

Separate determination of the hexon bases (arginin, lysine and histidin nitrogen) was carried out by VAN SLYKE'S method.

It may be here noted that the hydrolysed liquids were of yellow colour, which indicates that our preparations of the protamine sulphate were pure, as KOSSEL has already pointed out. In one case, however, with sciaenine sulphate, it was light brown, which was probably due to some impurities still adhering.

TABLE XXII.

	Lateoline sulphate.	Sciaenine sulphate.	Scombropine sulphate.	Serioline sulphate.	Scombromine sulphate.	Stereoline sulphate.
Total nitrogen.	21.297	21.089	21.186	21.950	21.175	22.974
Nitrogen soluble in HCl.	20.123	20.817	21.043	21.809	20.875	22.804
Nitrogen insoluble in HCl. (Melanin N)	0.174	0.272	0.143	0.141	0.300	0.170

	Lateoline sulphate.	Sciaenine sulphate.	Scombropine sulphate.	Serioline sulphate.	Scombromine sulphate.	Stereoline sulphate.
Ammonia nitrogen.	0.294	1.291	1.259	1.159	0.276	1.264
Total basic nitrogen.	14.935	11.618	16.782	16.712	15.772	19.766
{ 1. Arginine-nitrogen.	14.030	10.375	11.909	15.250	13.922	17.168
{ 2. Histidine-nitrogen.	0.902	0.688	4.384	0.199	1.553	1.923
{ 3. Lysine-nitrogen.	0.003	0.554	0.487	1.264	0.296	0.675
Amino-nitrogen.	3.258	2.956	1.783	3.649	3.782	1.458
Other-nitrogen.	2.636	5.052	1.219	0.289	1.045	0.067

Table XXIII and XXIV show the percentage of nitrogen in nitrogen compounds to the total nitrogen considered as 100, and the percentage of nitrogen of bases to the total basic nitrogen considered as 100.

TABLE XXIII.

	Lateoline sulphate.	Sciaenine sulphate.	Scombropine sulphate.	Serioline sulphate.	Scombromine sulphate.	Stereoline sulphate.
Total nitrogen.	100.00	100.00	100.00	100.00	100.00	100.00
Nitrogen soluble in HCl.	99.18	98.71	99.33	99.36	98.58	99.27
Nitrogen insoluble in HCl (Melanin N).	0.82	1.29	0.68	0.64	1.42	0.74
Ammonia nitrogen.	1.38	6.13	5.95	5.23	1.30	5.50
Total basic nitrogen.	70.13	55.09	79.23	76.13	74.49	86.02
{ 1. Arginine-nitrogen.	65.88	49.20	56.24	69.47	65.75	74.72
{ 2. Histidine-nitrogen.	4.24	3.26	20.70	0.91	7.34	8.37
{ 3. Lysine-nitrogen.	0.02	2.63	2.30	5.76	1.40	2.94
Amino-nitrogen.	15.30	14.02	8.42	16.62	17.86	6.35
Other nitrogen.	12.38	13.48	5.73	1.32	4.94	1.39

TABLE XXIV.

	Lateoline sulphate.	Sciaenine sulphate.	Scombropine sulphate.	Serioline sulphate.	Scombromine sulphate.	Stereoline sulphate.
Total basic nitrogen.	100.00	100.00	100.00	100.00	100.00	100.00
{ 1. Arginine-nitrogen.	93.94	89.54	70.86	91.00	88.11	86.67
{ 2. Histidine-nitrogen.	6.04	5.94	26.08	1.19	9.83	9.71
{ 3. Lysine-nitrogen.	0.28	4.78	2.90	7.54	1.87	3.41

TABLE XXV.

Comparison of the percentages of nitrogen in the sulphates calculated from the formula of the platinum chloride double salts with the percentages experimentally obtained.

	Lateoline.	Sciaenine.	Scombropine.	Serioline.	Scombromine.	Stereoline.
Total nitrogen in the sulphate.	21.30%	21.09%	21.19%	21.95%	21.18%	22.97%

	for 4 Lateo. + 11 SO ₄ H ₂	for Sci. + 2 SO ₄ H ₂	for Scombro. + 2 SO ₄ H ₂	for Serio. + 2 SO ₄ H ₂	for 6 Scombre. + 11 SO ₄ H ₂	for Ster. + 2 SO ₄ H ₂
Total nitrogen in the sulphate calculated from platonic chloride double salt.	21.40%	21.05%	21.40%	21.85%	21.27%	23.12%

TABLE XXVI.

The calculated percentages of hexon bases in protamines.

Arginine.	58.7%	42.7%	49.8%	62.0%	54.7%	69.4%
Histidine.	4.5%	3.4%	21.8%	0.95%	7.3%	9.2%
Lysine.	0.02%	3.5%	3.4%	8.6%	1.9%	4.6%

As Table XXVI shows, all the protamines generally contain relatively large quantities of arginine; the percentages of histidine are small, except in the case of scombropine. Among the known protamines there are some which yield neither lysine nor histidine on hydrolysis, but I found both bases in my new protamines. The numbers I obtained for mono-amino nitrogen may be larger than those, that might be obtained by the use of the ester-method. As I had no proper equipment for the employment of the ester-method, I did not try the isolation of mono-amino acids.

III. The Physiological Action of the New Protamines.

For five kinds of the new protamines I investigated their physiological actions; the research, however, was not complete.

COMPARATIVE EXPERIMENTS ON THE TOXIC POWER OF THE PROTAMINES.

I. Experiments with mice:—For the comparison of the toxic power I used a number of mice, into the backs of which protamine sulphate was injected hypodermically. Protamine sulphate was so dissolved in 0.8% common salt aqueous solution that 0.5 c.c. of the solution injected per 10 gr. of the weight of a mouse would contain a definite quantity of the sulphate.

A. Scombropine sulphate ;

(a) With 0.1 mgr. of the sulphate per 10 gr. of body-weight.

No.	Body-weight.	Time of injection.	Remarks.
1	12.3 gr. ♂	P.M. 1 ^h 4'.	Dyspnoea at 1 ^h 12'; alive on the following morning.
2	11.5 gr. ♂	P.M. 1 ^h 6'.	Restless.
3	12.2 gr. ♂	P.M. 1 ^h 8'.	"
4	12.1 gr. ♂	P.M. 1 ^h 10'.	"
5	11.0 gr. ♀	P.M. 1 ^h 11'.	"

(b) With 0.2 mgr. of the sulphate per 10 gr. of body-weight.

No.	Body-weight.	Time of injection.	Remarks.
1	12.0 gr. ♀	P.M. 2 ^h 21'.	Restless.
2	14.7 gr. ♀	P.M. 2 ^h 23'.	"
3	13.2 gr. ♀	P.M. 2 ^h 25'.	"
4	13.2 gr. ♀	P.M. 2 ^h 27'.	"
5	14.0 gr. ♀	P.M. 2 ^h 28'.	"

(c) With 0.5 mgr. of the sulphate per 10 gr. of body-weight.

No.	Body-weight.	Time of injection.	Remarks.
1	15.4 gr. ♀	P.M. 3 ^h 53'.	Anguish at 3 ^h 57'; alive the following morning.
2	11.9 gr. ♀	P.M. 3 ^h 55'.	Restless.
3	12.7 gr. ♀	P.M. 3 ^h 57'.	"

As there were no greater effects than those mentioned above with quantities less than 0.5 mgr. per 10 gr. of the body-weight of the mouse, I used 0.5 mgr. per 10 gr. for the other four protamines.

B. Stereoline sulphate;

No.	Body-weight.	Time of injection.	Remarks.
1	10.7 gr. ♂	A.M. 10 ^h 52'.	Restless, found dead on the following morning.
2	10.0 gr. ♂	A.M. 10 ^h 56'.	Restless, found dead on the following morning.
3	12.4 gr. ♂	A.M. 10 ^h 56'.	Restless.

C. Lateoline sulphate;

No.	Body-weight.	Time of injection.	Remarks.
1	10.9 gr. ♂	P.M. 1 ^h 41'.	Restless.
2	11.7 gr. ♂	P.M. 1 ^h 43'.	Restless, found dead on the following morning.
3	14.7 gr. ♂	P.M. 1 ^h 45'.	Restless.

D. Serioline sulphate;

No.	Body-weight.	Time of injection.	Remarks.
1	12.0 gr. ♂	P.M. 1 ^h 27'.	Restless.
2	11.2 gr. ♂	P.M. 1 ^h 28'.	"
3	12.2 gr. ♂	P.M. 1 ^h 32'.	"

E. Sciaenine sulphate;

No.	Body-weight.	Time of injection.	Remarks.
1	11.4 gr. ♂	P.M. 2 ^h 17'.	Restless.
2	11.5 gr. ♂	P.M. 2 ^h 18'.	Anguish at 2 ^h 25', alive on the following morning.
3	11.4 gr. ♀	P.M. 2 ^h 19'.	Restless.

It seems that stereoline and lateoline have relatively stronger toxic power on mice than the others.

II. Experiments with guinea pigs:—By substituting guinea pigs for mice I continued similar experiments. In this case I injected the protamine sulphate solution into the jugular vein. The sulphate solution was so pre-

pared that 1 c.c. of 0.8% common salt solution contained 0.02 gr. of the protamine salt, and a quantity of the solution was injected, so as to contain 0.05 gr. of the salt per kg. of the body-weight, except in the case of guinea pig No. 5.

A. Stereoline sulphate ;

No. 1. Guinea pig ♂, body-weight, 720 gr. Volume of the injected solution, 1.8 c.c. (0.05 gr. stereoline sulphate per kg. of body-weight). Time of injection, P.M. 2^h 26'. Death at 2^h 29 P.M. As soon as the solution was injected, the animal became restless with slow and irregular respiration, and paralysis of limbs set in; it excreted involuntarily, and at last died from paralysis of respiration after a few cramps. After death I found the heart still beating on opening the chest.

B. Lateoline sulphate ;

No. 2. Guinea pig ♂, body-weight 630 gr. Volume of the injected solution 1.58 c.c. (about 0.05 gr. lateoline sulphate per kg. of body-weight). Time of injection, 1^h 30' P.M. The animal twitched continuously from 1^h 38', was alive but restless on the following morning.

No. 3. Guinea pig ♂ body-weight 730 gr. Volume of the injected solution 1.8 c.c. (about 0.05 gr. lateoline sulphate per kg. of body-weight). Time of injection, 11^h 53' A.M. It twitched from 11^h 56' A.M. to 3^h 00' P.M. and was alive but restless on the following morning.

No. 4. Guinea pig ♀, body-weight, 720 gr. Volume of the injected solution 1.8 c.c. (about 0.05 gr. lateoline sulphate per kg. of body-weight.) Time of injection, 3^h 06' P.M. It twitched from 3^h 10' P.M. to evening, but was found alive though restless on the following morning.

No. 5. Guinea pig ♂, body-weight 580 gr. Volume of the injected solution 29 c.c. (about 0.1 gr. lateoline sulphate per kg of body-weight). Time of injection, 4^h 40' P.M. Death at 4^h 55' P.M. At 4^h 44' it began to twitch and seemed in great agony, motive paralysis followed with continued cramps of limbs until it died. After death I saw no movement of the heart on opening the chest.

With the three other protamines I saw no effect, except that the animals became restless and convulsed.

EFFECTS ON THE BLOOD-PRESSURE AND RESPIRATION.

Dissolving 0.5 gr. of protamine sulphate into 25 c.c. of aqueous solution of 0.8% common salt, I injected the solution into the jugular vein of the animals employed, rabbits and dogs. In some cases, aqueous solution of urethane was hypodermically injected or introduced into the stomach so as to quieten the animals. One of the carotid arteries was severed, a cannula was inserted into the end of the artery leading to the heart, and a manometre was connected with it. Then the wind-pipe was cut asunder, and the end of it, leading to the lung, was connected with MAREY's tambour, and a partial communication of the respiratory organ with the atmosphere was

established. Both the manometre and tambour were in connection with a kimograph.

I. Experiments on rabbits:—

TABLE XXVII.

February, 10th, 2576 (1916).

Rabbit ♂, body-weight 3000 gr. Lateoline sulphate, first injection 3.75 c.c. and second 3.75 c.c. (Total lateoline 0.037 gr. per kg. of body-weight.)

Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (3 gr.) sleep; tracheal cannula;

Time.	Blood pressure mm. Hg.	No. of pulse in 10 sec.	No. of respirations in 10 sec.	Remarks.
2h 36' 08''	73	43	41	Normal.
37 00''–38' 00	—	—	—	Injected lateoline sulphate 3.75 c.c. = 0.0555 gr. lateoline.
38' 30''	86		35	Could not count the pulse.
39' 57''	64		43	
41' 20''–42' 30''	—	—	—	Injected lateoline sulphate 3.75 c.c. = 0.0555 gr. lateoline.
43' 00''	40		42	
46' 40''	27		33	Amplitude of respiration wave somewhat irregular.
48' 00''	15		12	Wave amplitude small.
48' 30''	10			Aorta abdominalis compressed.
50' 30''	4		0	Respiration stopped.
52' 16''	0			Heart movement stopped; death.

At first the blood pressure rose somewhat, and the number of the respirations increased, but they fell after a while and the respiration stopped before the movement of the heart came to the end. After death, the coagulation of the blood seemed to be delayed.

TABLE XXVIII.

February 6th, 2576 (1916).

Rabbit ♀, body-weight 2250 gr. Sciaenine sulphate, first injection 5.6 c.c., second 3.0 c.c., and third 3.2 c.c. (Total sciaenine 0.0682 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (2.3 gr.) sleep; tracheal cannula.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	No. of re- spirations in 10 sec.	Remarks.
12 ^h 19' 20''	66	37	17	Normal.
20' 00''-21' 06''	—	—	—	Injected sciaenine sulphate 5.6 c.c. =0.0845 gr. sciaenine.
24' 00''	55	40	20	
26' 50''	32	40	23	Blood-pressure falling.
29' 50''-30' 52''	—	—	—	Injected sciaenine sulphate 3.0 c.c. =0.0453 gr. sciaenine.
34' 00''	31	41	—	
38' 00''	29	—	—	Respiration amplitude too small to be counted.
39' 15''-39' 50''	—	—	—	Injected sciaenine sulphate 2 c.c. =0.0302 gr. sciaenine.
42' 00''	33	40	—	
44' 20''	72	43	—	Blood pressure rising.
51' 00''	65	38	—	

The blood-pressure fell slightly at first, but pulse and respirations increased. It seemed that this protamine delays the coagulation of blood.

TABLE XXIX.

February 6th, 2576 (1916).

Rabbit ♀, body-weight 2200 gr. Sciaenine sulphate; first injection 5.5 c.c., second 2.25 c.c., third 2.25 c.c., and fourth 2.25 c.c. (Total sciaenine 0.0839 gr. per kg. of body-weight.) Manometre connected with left carotid; injection cannula in right jugular vein; urethane (2.2 gr.) sleep; tracheal cannula.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	No. of re- spirations in 10 sec.	Remarks.
3 ^h 59' 05''	97	51	9	Normal.
4 ^h 00' 05''-1' 35''	—	—	—	Injected sciaenine sulphate 5.5 c.c. =0.0829 gr. sciaenine.
3' 18''	39	47	17	
3' 22''-4' 08''	—	—	—	Injected sciaenine sulphate 2.25 c.c. =0.0339 gr. sciaenine.
5' 51''	12	14	17	
12' 45''	16	21	16	
15' 25''-16' 07''	—	—	—	Injected sciaenine sulphate 2.25 c.c. =0.0339 gr. sciaenine.
17' 00''	2	24	13	Respiration amplitude small.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	No. of re- spirations in 10 sec.	Remarks.
19' 00''	7	38	0	Respiration stopped.
21' 45''-22' 26''	—	—		Injected sciaenine sulphate 2.25 c.c. =0.0339 gr. sciaenine.
23' 00''	5			Pulse wave was too small to be counted.
26' 20''	0	0		Heart movement stopped; death.

At first the fall of the blood-pressure was considerable, and there was no change of the pulse, but the number of respirations was doubled, and the respiration stopped before the pulse. It seemed to delay the coagulation of blood.

TABLE XXX.

February 6th, 2576 (1916).

Rabbit ♀, body-weight 2250 gr. Scombropine sulphate; first injection 5.6 c.c., second 2.3 c.c. and third 3.3 c.c. (Total scombropine 0.0789 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (2.3 gr.) sleep; tracheal cannula.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	No. of re- spirations in 10 sec.	Remarks.
2 ^h 29' 50''	82	48	28	Normal.
32' 10''-33' 53''	—	—	—	Injected scombropine sulphate 5.6 c.c. =0.0852 gr. scombropine.
35' 30''	49	46	34	
40' 00''	23	47	38	
44' 00''-44' 55''	—	—	—	Injected scombropine sulphate 3 c.c. =0.046 gr. scombropine.
45' 35''	24	44	31	Respiration amplitude small.
52' 10''	30	46	28	
54' 15''-55' 15''	—	—	—	Injected scombropine sulphate 3 c.c. =0.0461 gr. scombropine.
56' 00''	14	50	16	Respiration weak.
3 ^h 00' 00''	38	40		Respiration very small amplitude.
1' 50''	21	10	3	Cramp.
3' 28''	7	0	0	Respiration stopped.
4' 00''	0	0	0	Heart movement stopped; death.

The fall of the blood-pressure after the injection was considerable, the

pulse was not much changed and the number of respirations increased somewhat. After death the coagulation of blood was delayed for about 30 minutes.

TABLE XXXI.

February 6th, 2576 (1916).

Rabbit ♀, body-weight 2400 gr. Serioline sulphate; first injection 3 c.c., the second 2 c.c. (Total serioline 0.0321 gr. per kg. of body-weight.) Manometer connected with left carotid artery; injection cannula in right jugular vein; urethane (2.4 gr.) sleep; tracheal cannula.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	No. of re- spirations in 10 sec.	Remarks.
10 ^h 52' 54''	101	46	12	Normal.
55' 29''-56' 27''	—	—	—	Injected serioline sulphate 3 c.c. =0.0459 gr. serioline.
58' 00''	70	41	10	Blood-pressure fell.
11 ^h 02' 00''	34	42	15	
3' 30''	31	43	15	Respiration weak, its amplitude small.
5' 40''	12	37	14	Blood-pressure fell considerably.
6' 00''	49	38	18	Cramp.
6' 20''	90	37	16	Respiration very weak.
9' 00''	92	32	13	
19' 50''	76	35	13	
22' 00''-22' 30''	—	—	—	Injected serioline sulphate 2 c.c. =0.0306 gr. serioline.
23' 27''	99	35	18	Restless.
23' 50''	100	28	0	Respiration stopped.
24' 00''	129	27	0	Blood-pressure fell.
26' 00''	0	0		Heart movement stopped; death.

At first the fall of the blood-pressure was considerable, but the pulse and respiration were not much changed. The respiration stopped before the pulse; the blood taken from the body did not coagulate for 24 hours.

TABLE XXXII.

February 7th, 2576 (1916).

Rabbit ♀, body-weight 2540 gr. Serioline sulphate; first injection 6 c.c., second 3 c.c. and third 3 c.c. (Total serioline 0.0724 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (2.5 gr.) sleep; tracheal cannula.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	No. of re- spirations in 10 sec.	Remarks.
5 ^h 2' 35''	90	37	9	Normal.
3' 00''-5' 00''	—	—	—	Injected serioline sulphate 6 c.c. =0.092 gr. serioline.
6' 00''	55	42	9	Respiration amplitude great.
12' 00''	36	38	10	
16' 00''	58	35	9	
18' 30''	—	—	—	Adrenaline injected.
18' 46''	118	32	14	Blood-pressure rose, and respiration was very weak.
19' 45''	65	37	13	
24' 00''-25' 30''	—	—	—	Injected serioline sulphate 3 c.c. =0.0459 gr. serioline.
27' 00''	55	35	8	Respiration amplitude small.
30' 00''	51	35	9	
31' 00''-32' 00''	—	—	—	Injected serioline sulphate 3 c.c. =0.0459 gr. serioline.
34' 45''	46	35	0	Respiration stopped.
36' 00''	86	26		
39' 12''	120	17		Blood-pressure rose.
42' 00''	51	32		
44' 00''	3	2		
45' 00''	0	0		Heart movement stopped; death.

At first the blood-pressure fell rapidly, the pulse and the respiration were not much changed, adrenaline being injected the blood vessel was contracted and the blood-pressure rose considerably.

TABLE XXXIII.

February 3rd, 2576 (1916).

Rabbit ♂, body-weight 2700 gr. Stereoline sulphate, first injection 3 c.c., second 2 c.c. (Total stereoline sulphate 0.0285 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; no urethane.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
11 ^h 48' 31''	91	50	Normal.
49' 00''-50' 00''	—	—	Injected stereoline sulphate 3 c.c. =0.0461 gr. stereoline.
50' 10''	116	40	Blood-pressure rose somewhat.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
53' 30''	58	40	Big fall of blood-pressure.
54' 40''	52	42	
56' 30''	42	41	
57' 30''	55	42	
58' 30''	—	—	Injected stereoline sulphate 2 c.c. =0.0308 gr. stereoline.
59' 30''	32	43	
12 ^h 2' 00''	42	42	
4' 00''	53	43	
6' 00''	60	41	Cramp.
8' 00''	78	36	
8' 40''	94	26	
10' 00''	86	32	
10' 10''	68	27	Respiration stopped.
11' 08''	18	16	
13' 00''	4	15	Heart movement stopped; death.
13' 34''	0	0	

TABLE XXXIV.
February 3rd, 2576 (1916).

Rabbit ♀, body-weight 2890 gr. Stereoline sulphate, first injection 2 c.c., second 3 c.c. (Total stereoline 0.0266 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; no urethane.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
10 ^h 36' 45''	125	41	Normal.
38' 00''-38' 30''	—	—	Injected stereoline sulphate 2 c.c. =0.0308 gr. stereoline.
40' 00''	124	35	
41' 8''-41' 30''	—	—	Injected stereoline sulphate 3 c.c. =0.0461 gr. stereoline.
43' 00''	122	30	
44' 00''	114	22	Cramp.
45' 00''	95	18	
45' 37''	75	23	Respiration stopped.
46' 00''	26	12	
46' 40''	4	4	Heart movement stopped; death.
47' 30''	0	0	

The fall of blood-pressure and the change of pulse were not great.

TABLE XXXV.

February 10th, 2576 (1916).

Rabbit ♀, body-weight 2500 gr. Stereoline sulphate, first injection 3.1 c.c., second 3.1 c.c. (Total stereoline 0.0382 gr. per kg. of body-weight.) Manometre connected with right carotid artery; injection cannula in left vein; urethane (2.5 gr.) sleep.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	No. of re- spirations in 10 sec.	Remarks.
6h 32' 20''	83	44	11	Normal.
33' 30''-34' 30''	—	—	—	Injected stereoline sulphate 3.1 c.c. =0.04768 gr. stereoline.
34' 50''	59	43	9	
38' 30''	52	42	11	
41' 30''-42' 30''	—	—	—	Injected stereoline sulphate 3.1 c.c. =0.04768 gr. stereoline.
45' 00''	91	39	8	
46' 55''	108	23	7	Pulse very weak.
50' 30''	120	26	0	Respiration stopped.
51' 30''	25	12		
54' 00''	0	0		Heart movement stopped; death.

The blood-pressure fell rapidly and the respiration stopped before the pulse. It seemed to delay the coagulation of the blood.

II. Experiments on dogs:—

TABLE XXXVI.

February 10th, 2576 (1916).

Dog ♂, body-weight 4400 gr. Lateoline sulphate, first injection 5.5 c.c., second 5.5 c.c. and third 5.5 c.c. (Total lateoline 0.0558 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (4.4 gr.) sleep.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
3h 44' 00''	148	31	Normal.
45' 00''-46' 50''	—	—	Injected lateoline sulphate 5.5 c.c. =0.0817 gr. lateoline.
48' 00''	19	23	Blood-pressure fell considerably.
40' 00''	22	22	

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
50' 00''	—	—	Injected adrenaline sulphate 0.5 c.c.
52' 20''	69	22	
55' 00''	106	27	Blood-pressure rose.
58' 00''—4h 00' 12''	—	—	Injected lateoline sulphate 5.5 c.c. =0.0817 gr. lateoline.
4h 1' 30''	132	35	
5' 50''	106	33	Amplitude of pulse wave was irregular.
16' 00''	129	36	
20' 00''—20' 40''	—	—	Injected lateoline sulphate 5.5 c.c. =0.0817 gr. lateoline.
22' 20''	120	36	

Though the fall of the blood-pressure was considerable, adrenaline being injected the contraction of blood vessels followed and the pressure rose rapidly. The blood of this dog did not coagulate.

TABLE XXXVII.

February 5th, 2576 (1916).

Dog ♂, body-weight 1650 gr. Sciaenine sulphate, first injection 4 c.c., second 4 c.c. (Total sciaenine 0.0732 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (1.6 gr.) sleep.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
2h 44' 10''	132	25	Normal.
45' 00''—46' 00''	—	—	Injected sciaenine sulphate 4 c.c. =0.0604 gr. sciaenine.
46' 30''	16	23	
48' 00''	13	20	Blood-pressure fell considerably.
51' 00''—51' 59''	—	—	Injected sciaenine sulphate 4 c.c. =0.0604 gr. sciaenine.
53' 00''	14	17	
55' 00''	37	18	Cramp.
56' 30''	87	18	Aorta abdominalis compressed.
57' 00''	53	7	
57' 30''	0	0	Heart movement stopped; death.

The fall of blood-pressure was considerable.

TABLE XXXVIII.

February 5th, 2576 (1916).

Dog ♂, body-weight 1300 gr. Scombropine sulphate 3 c.c. (Total scombropine 0.0354 gr. per kg. of body-weight.) Manometre connected with right carotid artery; injection cannula in left jugular vein; urethane (1.2 gr.) sleep.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
3 ^h 35' 00''	160	24	Normal.
36' 00''-37' 00''	—	—	Injected scombropine sulphate 3 c.c. =0.0459 gr. scombropine.
37' 20''	36	16	Blood-pressure fell considerably.
39' 30''	23	11	
40' 00''	0	0	Death.

The fall of blood-pressure was considerable.

TABLE XXXIX.

February 5th, 2576 (1916).

Dog ♀, body-weight 7400 gr. Serioline sulphate, 5 c.c. (Serioline 0.0765 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (7 gr.) sleep.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
5 ^h 11' 00''	140	19	Normal.
12' 00''-14' 30''	—	—	Injected serioline sulphate 5 c.c. =0.0765 gr. serioline.
15' 20''	47	28	
16' 30''	37	17	Blood-pressure fell considerably.
18' 10''	—	—	Injected adrenaline 0.5 c.c.
19' 00''	100	20	Blood-pressure rose considerably.
21' 30''	99	18	
34' 00''	106	24	

The blood-pressure fell considerably, on adrenaline being injected it rose at a rapid rate. The protamine delayed the coagulation of the blood.

TABLE XL.

February 10th, 2576 (1916).

Dog ♀, body-weight 4520 gr. Stereoline sulphate, first injection 5.6 c.c., second 1.4 c.c. and third 5.6 c.c. (Total stereoline 0.0330 gr. per kg. of body-weight.) Manometre connected with left carotid artery; injection cannula in right jugular vein; urethane (4.5 gr.) sleep.

Time.	Blood-pressure mm. Hg.	No. of pulse in 10 sec.	Remarks.
11 ^h 29' 40''	126	29	Normal.
30' 10''-31' 30''	—	—	Injected stereoline sulphate 5.6 c.c. =0.0861 gr. stereoline.
32' 10''	33	29	Blood-pressure fell considerably.
33' 00''	34	23	
33' 22''-33' 30''	—	—	Injected adrenaline 0.5 c.c.
34' 42''	148	12	Blood-pressure rose suddenly.
38' 00''	124	18	
41' 00''-41' 40''	—	—	Injected stereoline sulphate 1.4 c.c. =0.0215 gr. stereoline.
43' 00''	120	20	
44' 30''-46' 00''	—	—	Injected stereoline sulphate 5.6 c.c. =0.0861 gr. stereoline.
47' 00''	28	25	
48' 30''	63	22	
49' 17''	88	14	
57' 00''	110	23	
12 ^h 13' 00''	118	26	

The blood-pressure fell rapidly, but adrenaline being injected a contraction of the blood vessels resulted, and the blood-pressure rose considerably. This protamine sulphate delayed the coagulation of blood.

As the quantities of the new protamines were not sufficient further continuation of the experiments on these lines was not possible; we may state, however, that they are all toxic, though in less degree than those of the known protamines.

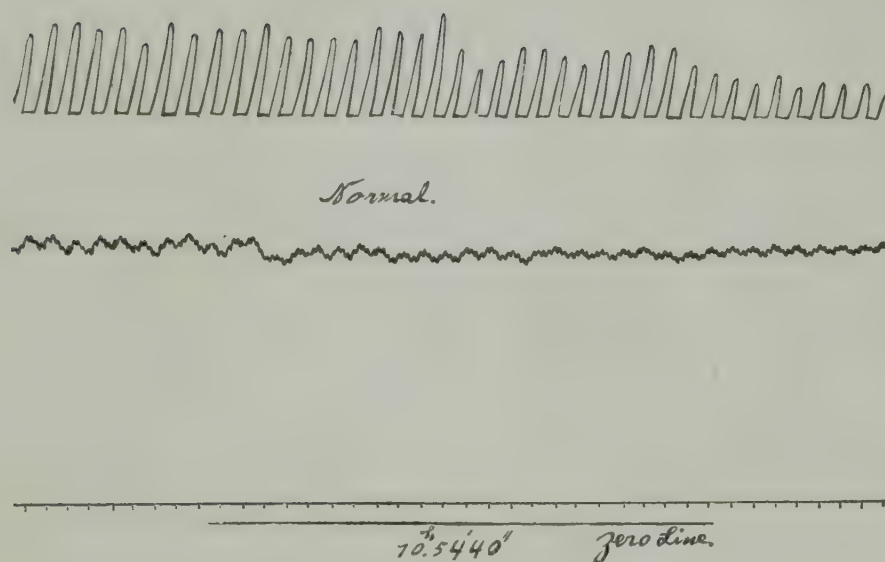


Fig. 1. Curves of the blood pressure and the respiration in the normal condition of a rabbit in the experiment described in Table XXXI. The upper is the respiration curve, and the lower, the blood pressure curve.

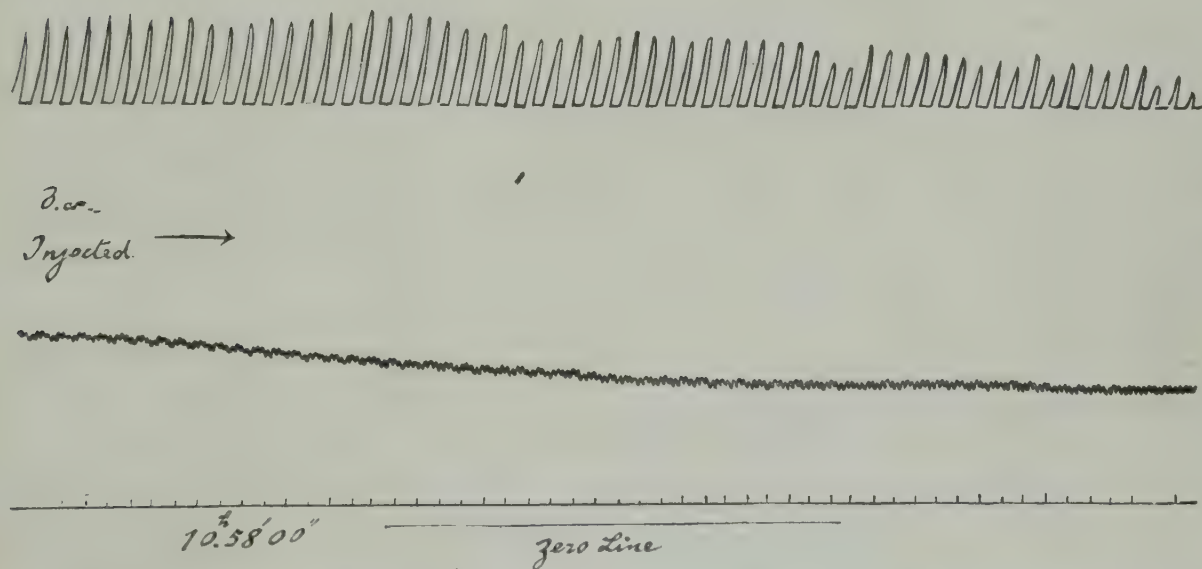


Fig. 2. Curve representing the fall of blood pressure after the injection of serioline sulphate in the same experiment as above.

TABLE

In the foregoing experiments of blood-pressure, the quantities of protamines and their the animal died, because in some cases, there were influences of adrenaline and of previous

No. and animal.	Protamines and their sulphates.	First injection.	Second injection.
No. 1. Rabbit.	Vol. of lateoline sulphate solution injected.	3.75 c.c.	3.75 c.c.
	Lateoline sulphate per kg. body-weight.	0.0250 gr.	0.0250 gr.
	Lateoline per kg. body-weight.	0.0186 gr.	0.0186 gr.
No. 2. Rabbit.	Vol. of sciaenine sulphate solution injected.	5.60 c.c.	3.00 c.c.
	Sciaenine sulphate per kg. body-weight.	0.0497 gr.	0.0266 gr.
	Sciaenine per body-weight.	0.0347 gr.	0.0201 gr.
No. 3. Rabbit.	Vol. of sciaenine sulphate solution injected.	5.50 c.c.	2.25 c.c.
	Sciaenine sulphate per kg. body-weight.	0.0500 gr.	0.0205 gr.
	Sciaenine per kg. body-weight.	0.0377 gr.	0.0154 gr.
No. 4. Rabbit.	Vol. of scombropine sulphate solution injected.	5.60 c.c.	3.00 c.c.
	Scombropine sulphate per kg. body-weight.	0.0497 gr.	0.0266 gr.
	Scombropine per kg. body-weight.	0.0381 gr.	0.0204 gr.
No. 5. Rabbit.	Vol. of serioline sulphate solution injected.	3.00 c.c.	2.00 c.c.
	Serioline sulphate per kg. body-weight.	0.0254 gr.	0.0166 gr.
	Serioline per kg. body-weight.	0.0194 gr.	0.0127 gr.
No. 6. Rabbit.	Vol. of serioline sulphate solution injected.	6.00 c.c.	3.00 c.c.
	Serioline sulphate per kg. body-weight.	0.0473 gr.	0.0236 gr.
	Serioline per kg. body-weight.	0.0362 gr.	0.0181 gr.
No. 7. Rabbit.	Vol. of stereoline sulphate solution injected.	3.00 c.c.	2.00 c.c.
	Stereoline sulphate per kg. body-weight.	0.0222 gr.	0.0148 gr.
	Stereoline per kg. body-weight.	0.0171 gr.	0.0114 gr.
No. 8. Rabbit.	Vol. of stereoline sulphate solution injected.	2.00 c.c.	3.00 c.c.
	Stereoline sulphate per kg. body-weight.	0.0138 gr.	0.0208 gr.
	Stereoline per kg. body-weight.	0.0106 gr.	0.0160 gr.
No. 9. Rabbit.	Vol. of stereoline sulphate solution injected.	3.10 c.c.	3.10 c.c.
	Stereoline sulphate per kg. body-weight.	0.0248 gr.	0.0248 gr.
	Stereoline per kg. body-weight.	0.0191 gr.	0.0191 gr.
No. 1. Dog.	Vol. of lateoline sulphate solution injected.	5.50 c.c.	5.50 c.c.
	Lateoline sulphate per kg. body-weight.	0.0250 gr.	0.0250 gr.
	Lateoline per kg. body-weight.	0.0186 gr.	0.0186 gr.
No. 2. Dog.	Vol. of sciaenine sulphate solution injected.	4.00 c.c.	4.00 c.c.
	Sciaenine sulphate per kg. body-weight.	0.0485 gr.	0.0485 gr.
	Sciaenine per kg. body-weight.	0.0366 gr.	0.0366 gr.

XII.

sulphates used were as follows, but the total quantities are not always mortal doses though injection of the sulphate solution.

Third injection.	Fourth injection.	Total.	Remarks.
—	—	7.50 c.c.	Injected lateoline sulphate, died.
—	—	0.0500 gr.	
—	—	0.0372 gr.	
2.00 c.c.	—	10.60 c.c.	Injected sciaenine sulphate, alive.
0.0177 gr.	—	0.0940 gr.	
0.0134 gr.	—	0.0682 gr.	
2.25 c.c.	2.25 c.c.	12.25 c.c.	Injected sciaenine sulphate, died.
0.0205 gr.	0.0205 gr.	0.1115 gr.	
0.0154 gr.	0.0154 gr.	0.0839 gr.	
3.00 c.c.	—	11.60 c.c.	Injected scombropine sulphate, died.
0.0266 gr.	—	0.1029 gr.	
0.0204 gr.	—	0.0789 gr.	
—	—	5.00 c.c.	Injected serioline sulphate, died.
—	—	0.0420 gr.	
—	—	0.0321 gr.	
3.00 c.c.	—	12.00 c.c.	Injected serioline sulphate and adrenaline ($1/10000$ solution), died.
0.0236 gr.	—	0.0945 gr.	
0.0181 gr.	—	0.0724 gr.	
—	—	5.00 c.c.	Injected stereoline sulphate, died.
—	—	0.0370 gr.	
—	—	0.0285 gr.	
—	—	5.00 c.c.	Injected stereoline sulphate, died.
—	—	0.0346 gr.	
—	—	0.0266 gr.	
—	—	6.20 c.c.	Injected stereoline sulphate, died.
—	—	0.0496 gr.	
—	—	0.0382 gr.	
5.50 c.c.	—	16.50 c.c.	Injected lateoline sulphate and adrenaline ($1/10000$ solution), alive.
0.0250 gr.	—	0.0750 gr.	
0.0186 gr.	—	0.0558 gr.	
—	—	8.00 c.c.	Injected sciaenine sulphate, died.
—	—	0.0970 gr.	
—	—	0.0732 gr.	

No. and animal.	Protamines and their sulphates.	First injection.	Second injection.
No. 3. Dog.	Vol. of scombropine sulphate solution injected.	3.00 c.c.	—
	Scombropine sulphate per kg. body-weight.	0.0462 gr.	—
	Scombropine per kg. body-weight.	0.0354 gr.	—
No. 4. Dog.	Vol. of serioline sulphate solution injected.	5.00 c.c.	—
	Serioline sulphate per kg. body-weight.	0.0135 gr.	—
	Serioline per kg. body-weight.	0.0103 gr.	—
No. 5. Dog.	Vol. of stereoline sulphate solution injected.	5.60 c.c.	1.40 c.c.
	Stereoline sulphate per kg. body-weight.	0.0248 gr.	0.0062 gr.
	Stereoline per kg. body-weight.	0.0191 gr.	0.0048 gr.

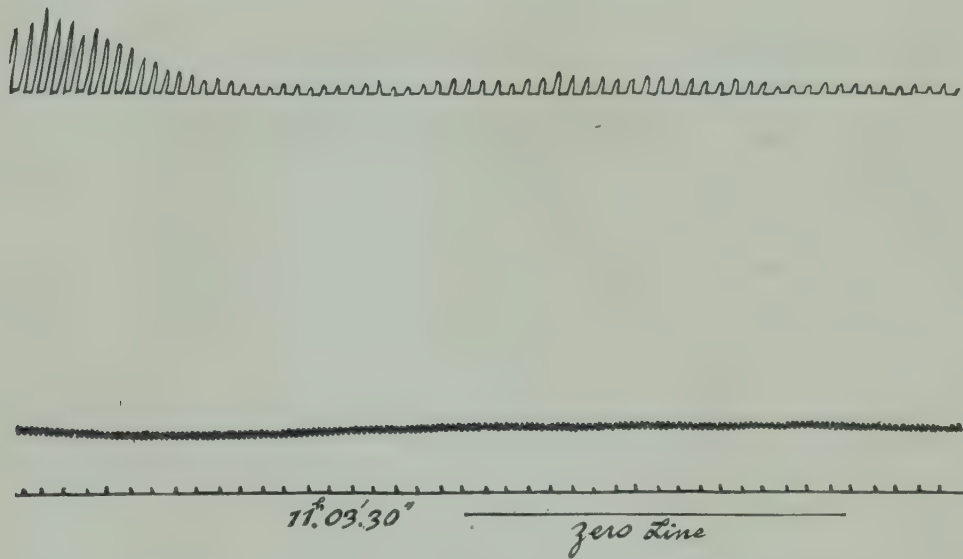


Fig. 3. The lowest part of the blood pressure curve after the injection of serioline sulphate in the same experiment, the respiration is weak, its amplitude being very small.

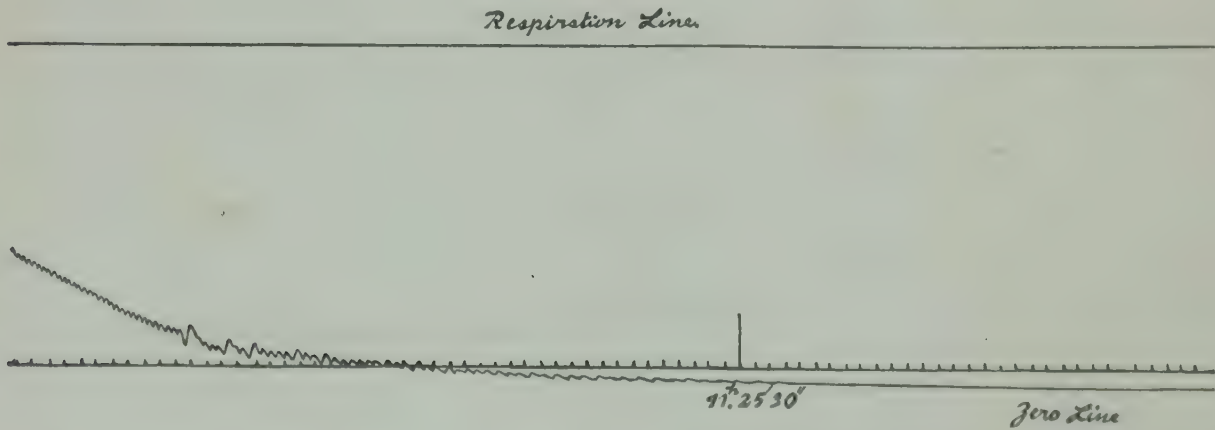


Fig. 4. Curve about the time of death in the same experiment, the respiration stopped long before.

Third injection.	Fourth injection.	Total.	Remarks.
—	—	3.00 c.c.	Injected scombropine sulphate, died.
—	—	0.0462 gr.	
—	—	0.0354 gr.	
—	—	5.00 c.c.	Injected serioline sulphate and adrenaline ($\frac{1}{10000}$ solution), alive.
—	—	0.0135 gr.	
—	—	0.0103 gr.	
5.60 c.c.	—	12.60 c.c.	Injected stereoline sulphate and adrenaline ($\frac{1}{10000}$ solution), alive.
0.0248 gr.	—	0.0558 gr.	
0.0191 gr.	—	0.0330 gr.	

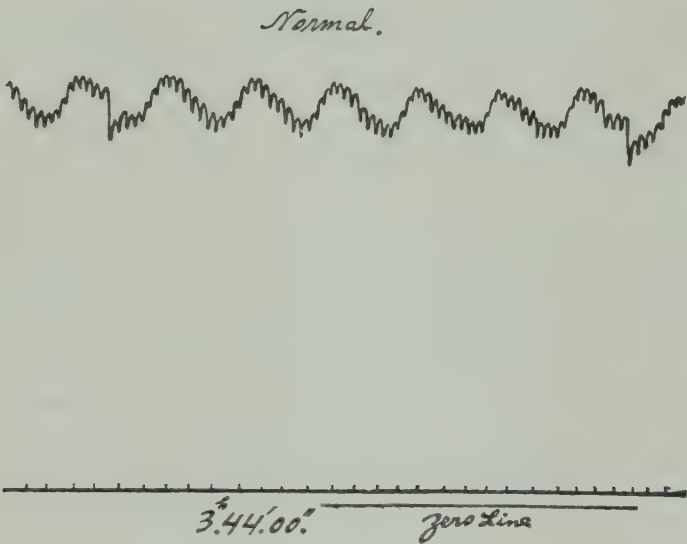


Fig. 5. Curve of blood pressure in the normal condition of a dog in the experiment described in Table XXXVI.

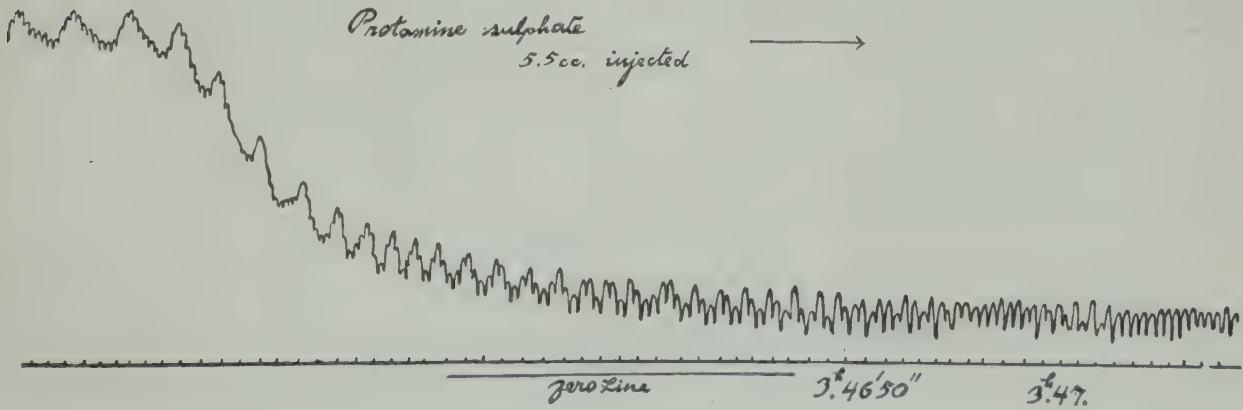


Fig. 6. The fall of blood pressure after injection of lateoline sulphate in the same experiment as above.

As regard the injection of protamine sulphate, we observe that the effect of serioline on rabbits, especially with reference to the fall of blood pressure, is greater than that of the other protamines, and all five protamines act powerfully on dogs. In mortal cases respiration stopped before the pulse. From the fact that after injection of protamines the injection of adrenaline and the compression of the aorta abdominalis raised the blood-pressure, it seems that every protamine causes the dilatation of blood vessels, resulting in a fall of blood-pressure.

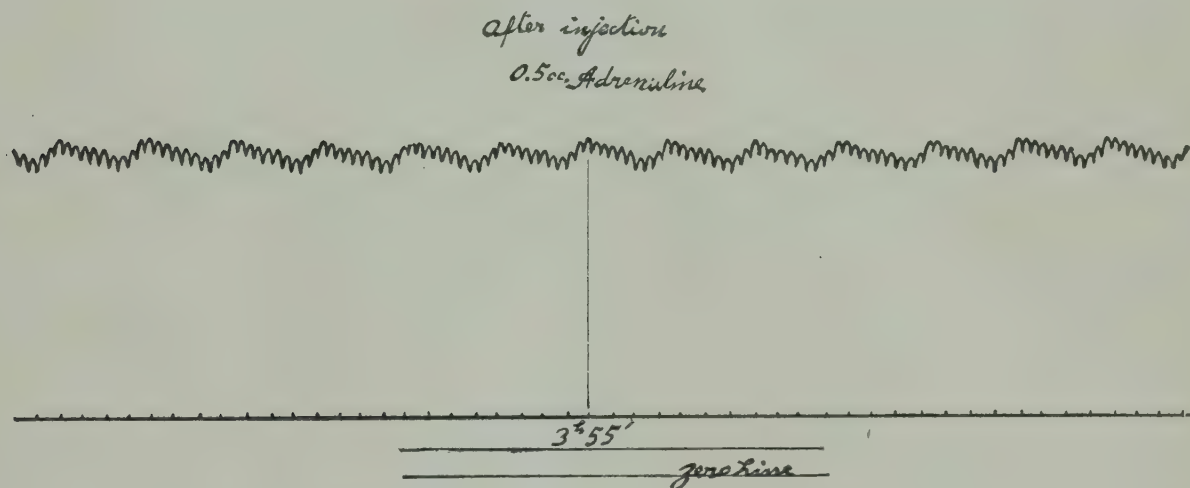


Fig. 7. The highest part of the blood pressure curve after the injection of adrenaline in the same experiment.

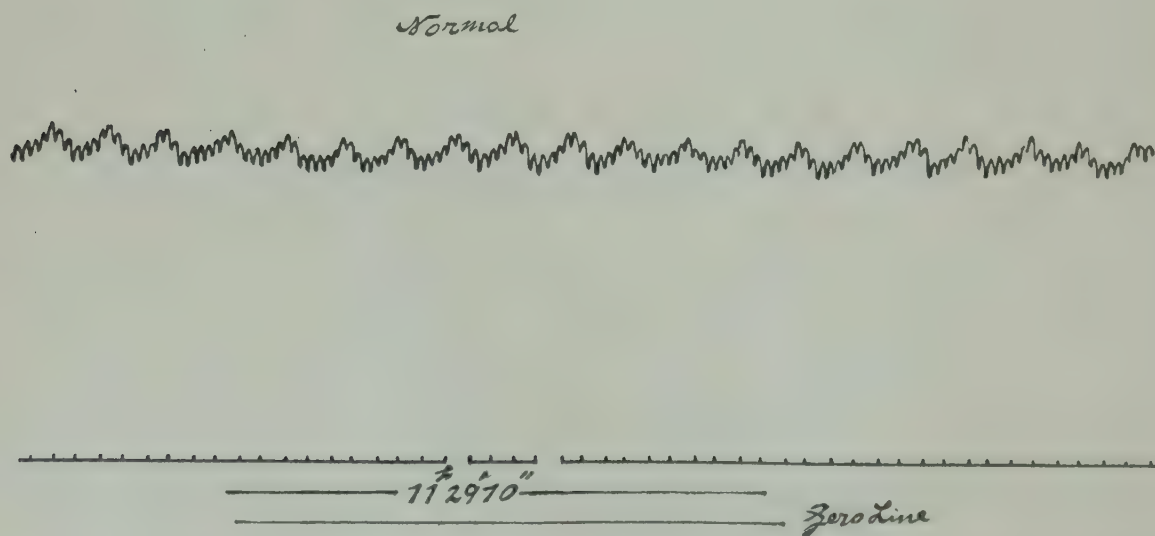


Fig. 8. Curve of blood pressure in the normal condition of a dog in the experiment described in Table XL.

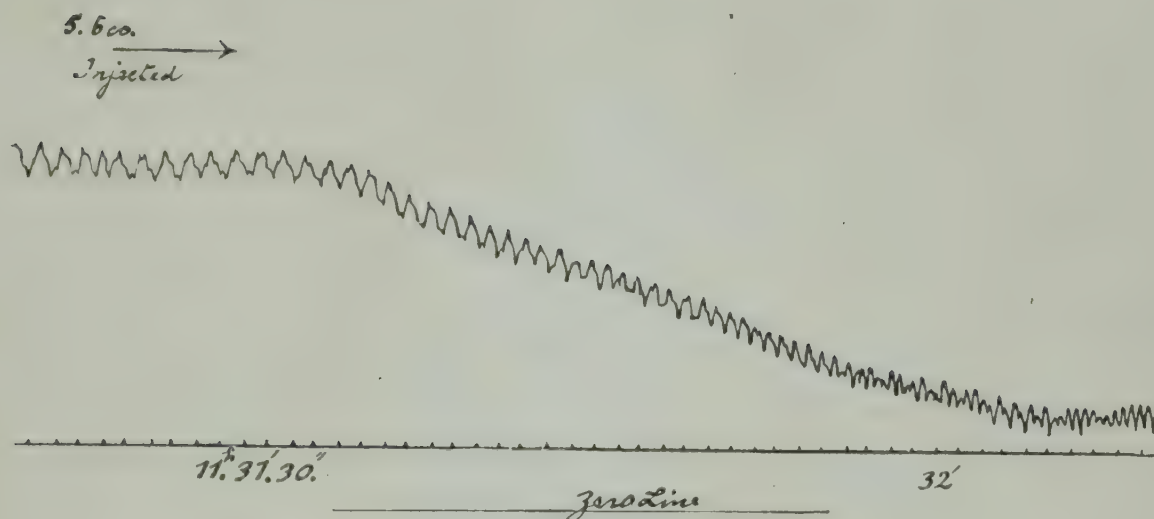


Fig. 9. The blood pressure falling after the injection of stereoline sulphate in the same experiment as above.

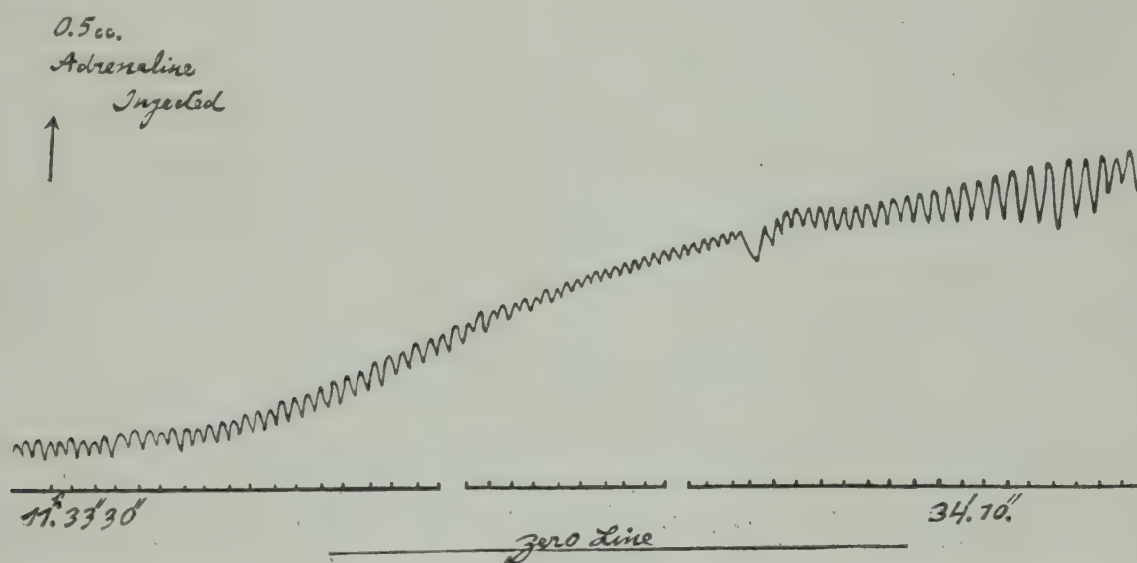


Fig. 10. Curve of blood pressure showing the rise after the injection of adrenaline in the same experiment.

Though I made no special study on the coagulation of the blood, yet I observed during the investigation on the blood pressure that the coagulation was generally delayed after the injection of the protamines; this was especially the case with serioline on rabbits and with lateoline on dogs.

IV. Summary.

The outlines of the present researches are as follows:—

1. Protamines are isolated from the sperm of some fishes caught in Japan.

2. The properties of the isolated protamines are analogous to those of the known protamines.

3. Arginine is the chief product of hydrolysis of the new protamines, though its percentage is generally lower than those in the known protamines.

4. Injection of the protamines produces toxic effects on mice, guinea pigs, rabbits and dogs.

5. On injecting the new protamines into the circulation system of rabbits and dogs, the blood pressure is considerably depressed. The new protamines seem to cause the dilatation of blood vessels. Further, they seem to hinder or to delay the coagulation of blood.

In conclusion, I express my hearty thanks to Prof. Dr. H. HAYASHI of the Medical College, Tokio Imperial University, from whom I got kind permission to work in his pharmacological laboratory and according to whose valuable suggestions I was able to carry out the physiological experiments with my protamines. I am also greatly indebted to his assistant, Dr. S. SHIMIZU, who was kind enough to devote much of his valuable time and labor to help me.

The first part of this paper, i. e. the chemical studies on the protamines, was done in the chemical laboratory of the Fishery Institute of the Department of Agriculture and Commerce under the collaboration of my assistants Messrs. S. YAMAMOTO and T. ASAKURA, to whom I express here my sincere thanks.

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